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VITAMINA D EM DOENÇAS TIREOIDIANAS AUTOIMUNES E RELAÇÃO COM PERFIL HORMONAL TIREOIDIANO E ATIVIDADE INFLAMATÓRIA DE CÉLULAS Th1, Th2 e Th17

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RESUMO

Introdução: O papel imunomodulador da vitamina D tem sido amplamente pesquisado. Estudos recentes demonstram haver relação entre insuficiência de vitamina D e presença de doenças tireoideanas autoimunes (DTAs) como Tireoidite de Hashimoto (TH) e Doença de Graves (DG). É possível que a vitamina D promova inibição do processo autoimune em diferentes estágios destas doenças. Nossos objetivos foram estudar a prevalência de insuficiência de vitamina D e a relação de suas concentrações séricas com marcadores de função tireoidiana e atividade inflamatória de linfócitos Th1, Th2 e Th17.

Material e métodos: Amostras de sangue foram coletadas de 88 pacientes com TH, 105 pacientes com DG seguidos em hospital universitário e 71 indivíduos sem DTAs, com idade entre 18 e 65 anos. Foram realizadas dosagens de vitamina D total (25OHD), tireotrofina (TSH), tiroxina livre (T_4L), triiodotironina livre (T3L), cálcio, fósforo, paratormônio (PTH), anticorpo antitireoperoxidase (AcTPO), anticorpo antitireoglobulina (AcTG) e antirreceptor de TSH (TRAb), além de citocinas produzidas por células Th1 (IL-2, IFN-γ, TNF-α), Th2 (IL-4, IL-5) e Th17 (IL-17). O volume tireoidiano foi estimado por ultrassonografia. Dados sobre peso, altura, índice de massa corporal, paridade e tempo de diagnóstico dos pacientes foram coletados diretamente por entrevista. O nível de significância estatística adotado foi 5%.

Resultados: Maior prevalência de insuficiência de vitamina D foi encontrada em pacientes com DG comparativamente aos controles ($p = 0.0078$), associando-se ao uso de tionamidas e a menores concentrações de IFN-γ e T4L, além de maiores volumes tireoideanos. No grupo TH não houve diferença entre as concentrações da vitamina comparado aos controles ($p = 0.1024$). Houve uma correlação positiva entre níveis de vitamina D e concentrações de interleucinas TNF-α, IL-5 e IL-17 nos pacientes do grupo TH.

Conclusões: Demonstramos maior prevalência de insuficiência de vitamina D em pacientes com DG em relação a indivíduos de um grupo controle saudável, o que

não ocorreu no grupo TH quando comparado ao grupo controle. Associação com tionamidas sugere envolvimento da insuficiência de vitamina D na atividade de doença. Menores concentrações de T4 livre, ainda que dentro dos valores de referência, se evidenciaram como fator preditor de insuficiência de vitamina D, indicando a importância da manutenção do eutireoidismo no adequado *status* de vitamina D tanto em DG quanto em TH. Apenas nos pacientes e não no grupo de controle houve relação entre vitamina D e interleucinas, indicando o envolvimento entre a vitamina e o processo imunológico nas DTAs.

Palavras-chave: Tireoide; Autoimunidade; Interleucinas.

ABSTRACT

Introduction: The immunomodulatory role of vitamin D has been extensively researched. Recent studies have demonstrated a relationship between vitamin D insufficiency and the presence of autoimmune thyroid disease (AITDs) such as Hashimoto's Thyroiditis (HT) and Graves' Disease (GD). It is possible that vitamin D promotes inhibition of the autoimmune process at different stages of these diseases. Our objectives were to study the prevalence of vitamin D insufficiency and the relation of its serum concentrations with markers of thyroid function and inflammatory activity of Th1, Th2 and Th17 lymphocytes.

Material and methods: Blood samples were collected from 88 patients with HT, 105 patients with GD followed in a university hospital and 71 individuals without AITDs, aged between 18 and 65 years. It was performed measurements of serum total 25OH vitamin D, thyrotropin (TSH), free thyroxine (FT4), free triiodothyronine (FT3), calcium, phosphorus, parathyroid hormone (PTH), thyroid peroxidase antibodies (TPOAb), thyroglobulin antibodies (TGAb) in addition to cytokines produced by Th1 (IL-2, IFN- γ,TNF-α), Th2 (IL-4, IL-5), and Th17 cells (IL-17). Thyroid volume was estimated by ultrasonography. Data on weight, height, body mass index, parity and time of diagnosis of the patients were collected directly by interview. The level of statistical significance was 5%.

Results: Higher prevalence of vitamin D insufficiency was found in patients with GD compared to controls ($p = 0.0078$) associated with the use of thionamides and lower concentrations of IFN-γ and FT4 in addition to higher thyroid volumes. In the HT group there was no difference between vitamin concentrations compared to controls ($p = 0.1024$). There was a positive correlation between vitamin D levels and concentrations of interleukins TNF-α, IL-5 and IL-17 in HT patients.

Conclusions: We demonstrated a higher prevalence of vitamin D insufficiency in patients with GD in relation to individuals from a healthy control group, which did not occur in the HT group when compared to the control group. Association with thionamides suggests involvement of vitamin D insufficiency in disease activity. Lower concentrations of free T4, although within the reference values, were

evidenced as a predictor of vitamin D insufficiency, indicating the importance of maintaining euthyroidism in the adequate vitamin D status in both GD and HT. There was a relationship between vitamin D and interleukins only in the patients and not in the control group, indicating the involvement between the vitamin and the immune process in the AITDs.

Keywords: Thyroid; Autoimmunity; Interleukins.

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LISTA DE ABREVIATURAS

AcTG: Anticorpo antitireoglobulina
AcTPO: Anticorpo antitireoperoxidase
APC: Célula apresentadora de antígeno
Ca: Cálcio
DBP: Proteína de Ligação da vitamina D
DG: Doença de Graves
DM-1: Diabetes Mellitus tipo 1
DM-2: Diabetes Mellitus tipo 2
DTA: Doença Tireoideana Autoimune
FGF-23: Fator de Crescimento de fibroblastos 23
IL: Interleucina
IMC: Índice de Massa Corporal
P: Fósforo
PTH: Paratormônio
T4 livre: Hormônio tiroxina livre
TCD4+: Linfócito T CD4
TCD8+: Linfócito T CD8
TG: Tireoglobulina
TH: Tireoidite de Hashimoto
Th: Linfócito T helper
TPO: Tireoperoxidase
TRAb: Anticorpo antirreceptor de TSH
Treg: Linfócito T regulador
TSH: Tireotrofina (Hormônio estimulador da tireoide)
TSH-R: Receptor de TSH
25OHD: 25-hidroxi-vitamina D; calcidiol
1,25(OH)2D: 1,25-dihidroxi-vitamina D; Calcitriol
VDR: Receptor de Vitamina D

SUMÁRIO

APRESENTAÇÃO GERAL

A vitamina D exerce suas principais ações no metabolismo do cálcio e na saúde óssea. Nos últimos anos, muitos estudos evidenciaram o papel da vitamina D na fisiopatologia de doenças autoimunes, cuja incidência vem aumentando continuamente. A deficiência de vitamina D associa-se às principais doenças autoimunes como Diabetes Mellitus tipo 1, Esclerose Múltipla, Artrite Reumatoide, Lupus Eritematoso, Doenças Tireoidianas Autoimunes, entre outros. A associação com neoplasias e Diabetes Mellitus tipo 2 também tem sido estudada.

A causa da perda da tolerância imunológica a autoantígenos ainda não está bem esclarecida. Sabe-se que fatores ambientais assim como polimorfismos genéticos, entre outros, favorecem o surgimento de doenças autoimunes em indivíduos suscetíveis, mas o evento que desencadeia tais patologias ainda não está bem definido.

A deficiência de vitamina D vem sendo apontada como um fator ambiental atuante no aumento da prevalência de doenças autoimunes agindo na sua patogênese. Estudos realizados em modelos animais demonstraram que administração de análogos da vitamina D exerceriam ação preventiva capaz de modificar a incidência ou o curso de doenças autoimunes. Porém, existem poucos estudos no homem evidenciando a obtenção de melhor controle metabólico realizados em pacientes com Diabetes Mellitus tipo 1 que receberam vitamina D. Há estudos correlacionando influência de altos níveis de vitamina D na redução da incidência de Esclerose Múltipla. Ainda são necessários estudos randomizados e controlados para se avaliar o melhor componente e dose a ser administrada para obter a eficácia clínica desejada no controle da autoimunidade, além de tempo de suplementação e efeitos colaterais envolvidos.

Considerando-se o fato de Tireoidite de Hashimoto (TH) e Doença de Graves (DG) estarem entre as doenças endócrinas autoimunes mais comuns e que a relação entre função tireoidiana e insuficiência de vitamina D ainda não está completamente elucidada, desenvolvemos um estudo com objetivo de comparar níveis de vitamina D em pacientes com DG, TH e um grupo de controle correlacionando as concentrações da vitamina a testes de função tireoidiana,

concentrações de interleucinas Th1, Th2, Th17 e autoanticorpos antitireoide. O presente trabalho visa contribuir com o fornecimento de dados para o desenvolvimento de novos estudos sobre possíveis tratamentos futuros.

INTRODUÇÃO

1. Autoimunidade tireoidiana

1.1 Fisiopatologia

As doenças tireoidianas autoimunes (DTAs) são representadas sobretudo pela Tireoidite de Hashimoto (TH) e pela Doença de Graves (DG), principais responsáveis pela disfunção tireoidiana clínica ou subclínica. O ataque autoimune da tireoide ocorre através da infiltração da glândula por linfócitos T e B com a participação já bem documentada de citocinas inflamatórias e presença de anticorpos antitireoidianos circulantes (1-6).

Nas DTAs há quebra de tolerância aos autoantígenos tireoidianos. A autoimunidade desenvolve-se através da quebra de tolerância a um autoantígeno, resultando no desenvolvimento de resposta imune. Vários são os fatores associados a esta quebra de tolerância como infecção, predisposição genética, drogas como alguns quimioterápicos, influências do meio ambiente, concentração de iodo e de selênio, entre outros. Na glândula tireoide existem alguns antígenos envolvidos na autoimunidade dentre os quais destacam-se a tireoglobulina (TG), tireoperoxidase (TPO) e receptor de TSH (5,7-10).

O ataque imune nas DTAs se dá através da interação entre imunidade celular e imunidade humoral em que uma delas prevalece. O tipo de apresentação do antígeno, a diferenciação específica em células T ou B, a ação do linfócito T regulador além da interação com componentes ambientais e genéticos define o padrão de doença estabelecida (11 - 16).

Na TH ocorre um defeito genético na função da célula T supressora (Treg) e as células T CD4 + (auxiliares/helper) tipo 2 (Th2) não são inativadas, tornando-se livres para promover a ativação de linfócitos B responsáveis pela produção de anticorpos contra o tecido tireoideano. Concomitantemente, as células Th (helper/auxiliares) tipo 1 (Th1) e tipo 2 (Th2) produzem citocinas que induzem os tireocitos a expressarem antígenos de superfície HLA-DR tornando-os

apresentadores de antígenos e suscetíveis ao ataque imunológico. A reação imune mediada por linfócitos T citotóxicos e macrófagos é predominante na TH, observando-se presença marcante de citocinas Th1 como IL-2; IFN-γ e TNF-α além de IL-17, pertencente ao grupo Th17. Há destruição direta com apoptose das células foliculares e acúmulo local de linfócitos T e B associado à fibrose do tecido (Figura 1) (1, 2, 6, 13, 17).

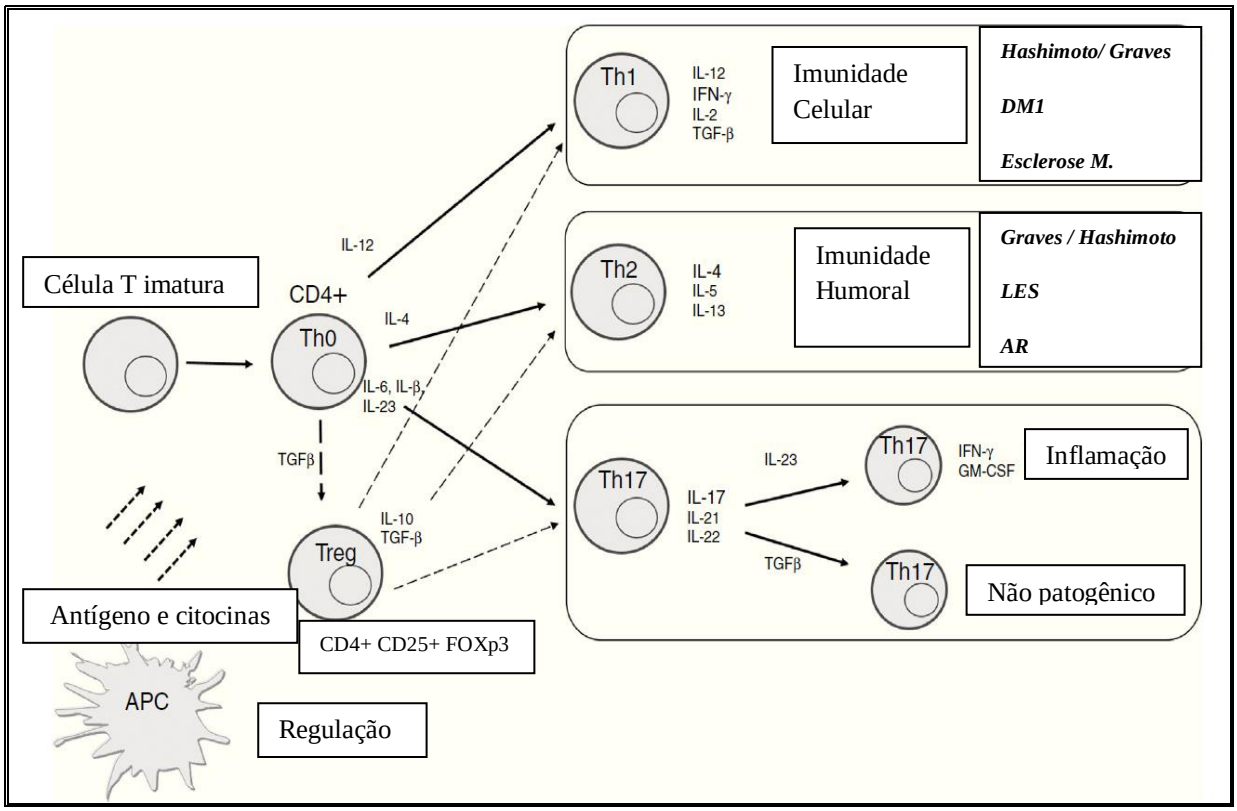
A Doença de Graves (DG) decorre principalmente da ativação da imunidade humoral via células Th2 com presença marcante das citocinas IL-4 e IL-5, produção de anticorpos antirreceptor de TSH (TRAb) estimuladores levando à proliferação e hipertrofia celular tireoidiana e hipertireoidismo (Figura 1) (1, 4, 7, 11, 13).

As DTAs ocorrem com frequência significativamente maior no sexo feminino em proporção de 5 até 10 mulheres para cada homem. Vários estudos foram desenvolvidos para explicar tal prevalência. A relação da paridade com maior incidência de DTAs, além da presença de microquimerismo fetal no sangue e tecido tireoidiano de mulheres com DTAs são apontados como possíveis responsáveis por este aumento de prevalência no sexo feminino (9, 18).

Sabe-se que o microquimerismo fetal resulta da persistência de tecidos fetais na circulação materna após o parto comportando-se como autoantígenos que estimulam a resposta imune materna podendo então deflagrar doenças autoimunes na mãe. Há estudos indicando que a inativação do cromossomo X pode ocorrer no sexo feminino durante a embriogênese, levando possivelmente à inativação de genes codificadores de determinados autoantígenos relacionados ao desenvolvimento de autotolerância que estariam associados a este cromossomo sexual (9,18).

Há estudos que citam a desregulação no receptor de vitamina D (VDR) presente no endométrio. Evidências demonstram o importante papel de alguns fatores como a vitamina D na programação do metabolismo e da resposta imunológica em períodos críticos do desenvolvimento fetal e pós-natal. Alterações permanentes em muitos processos fisiológicos nesta fase podem modificar padrões de expressão gênica com influência em fenótipos (mecanismos epigenéticos) e com consequente risco para o desenvolvimento de fenótipos de doenças. Porém, ainda

se desconhece a real dependência destas alterações ao tempo de exposição a um ambiente materno com deficiências como a hipovitaminose D (9,18 - 20).



APC: Célula apresentadora de antígeno; Treg: Linfócito T regulador; Th1: Linfócito T helper tipo 1; Th2: Linfócito T helper tipo 2; Th17: Linfócito T helper tipo 17

Figura 1: Principais mecanismos envolvidos no desenvolvimento da doença tireoidiana autoimune.

(Fonte: Adaptado de Ramos-Leví et al, 2016)

2. Imunidade e citocinas

Os linfócitos T são subdivididos de acordo com sua função em linfócitos T citotóxicos (TCD8), que expressam em sua superfície a proteína CD8, T-*helper* ou auxiliar (TCD4), que expressam em sua superfície a proteína CD4, e linfócitos T reguladores (T-reg). As células T são ativadas após exposição a antígenos específicos sofrendo posterior diferenciação no timo que através de mecanismos

centrais de imunotolerância detecta linfócitos T autorreativos. Em alguns indivíduos predispostos, certos linfócitos escapam deste controle tímico, proliferando-se normalmente na circulação e posteriormente deflagrando resposta autoimune. As células apresentadoras de antígenos são representadas normalmente por células dendríticas, macrófagos e de modo aberrante na DTA por células foliculares da tireoide, associando-se assim ao desenvolvimento da DG e da TH (1,4,5,7,11).

As células T reguladoras, também denominadas linfócitos T supressores, têm como função impedir o ataque do sistema imune a autoantígenos. Estas células atuam sobre os linfócitos TCD8 e TCD4. As células TCD4 auxiliam outras células como os linfócitos TCD8, macrófagos ou linfócitos B na resposta imune. As células TCD8 são responsáveis pela destruição direta dos patógenos através do ataque a células infectadas pelos mesmos, estando envolvidas na resposta imune a infecções virais, ataque a células tumorais e rejeição a transplantes (1,4).

As células T-*helper* (Th) participam de todas as respostas imunes antígeno específicas. O microambiente em que as células Th não ativas se desenvolverão determina o tipo de resposta imune predominante: Th1 ou Th2, e eles se autorregulam. As células Th1 secretam citocinas inflamatórias como o interferon-γ (IFN-γ), a interleucina-2 (IL-2) e o fator de necrose tumoral alfa (TNF-α), essenciais para a resposta imune mediada por células. As células Th2 secretam as citocinas inflamatórias IL-4 e IL-5, importantes para a resposta imune mediada por anticorpos. Outro subtipo de células Th, as células Th-17, também composto por linfócitos T CD4 +, mostra envolvimento na fisiopatologia de doenças autoimunes e secretam as interleucinas IL-17 e IL-23. Adicionalmente, há o grupo de células definido como Th0, que produz tanto citocinas de Th1 quanto de Th2 (1,2,4,6,11-14).

Os linfócitos B participam ativamente do processo de autoimunidade através da produção de anticorpos, a chamada imunidade humoral. Sintetizam anticorpos contra antígenos específicos reconhecidos via indução decorrente da apresentação de fragmentos destes antígenos aos linfócitos T (1,4).

As Doenças Tireoidianas Autoimunes (DTAs) são marcadas tanto pela presença de imunidade celular com infiltração e destruição da glândula por linfócitos T quanto por imunidade humoral com a presença de linfócitos B ativos produzindo anticorpos específicos contra os tecidos tireoidianos. O envolvimento das citocinas

Th1, Th2 e Th17 na fisiopatologia das DTAs encontra-se bem estabelecido, além da evidente redução da atividade dos linfócitos T reguladores com consequente prejuízo nos mecanismos de autotolerância nestas doenças (1,2,4,6,7,11-15).

Estudos mostram que a presença de concentrações elevadas de autoanticorpos contra antígenos tireoidianos desempenha um papel importante no estímulo da produção de citocinas por linfócitos T e monócitos nas DTAs, além de facilitar a proliferação de células TCD4 nestes indivíduos. As células T regulatórias assim como algumas células B e monócitos produzem citocinas específicas, como IL-10, que exercem efeito protetor no desenvolvimento da autoimunidade (1,4,7,11).

3. Doenças tireoidianas autoimunes

3.1Tireoidite de Hashimoto

3.1.1 Definição

Tireoidite crônica de Hashimoto (TH), igualmente conhecida como tireoidite crônica autoimune ou tireoidite linfocítica crônica, foi descrita em 1912 por Hakaru Hashimoto e caracteriza-se por infiltrado linfocitário difuso na glândula tireoide. Principal causa de hipotireoidismo em áreas suficientes em iodo, tem sua incidência estimada em 0.3 a 1.5 casos/1000 pessoas/ano, sendo mais freqüente no sexo feminino e com incidência mais alta entre 45 e 65 anos de idade. Apresenta etiologia multifatorial, notando-se ativação de genes específicos, alterações imunológicas marcadas pela produção de autoanticorpos reativos a antígenos tireoidianos, ativação de grupos específicos de citocinas, além de influências do meio ambiente. A disfunção da glândula tireoidiana pode apresentar-se como clínica ou subclínica, de acordo com o grau de comprometimento do parênquima (1, 2, 6, 17, 21).

A glândula exibe textura heterogênea à ultrassonografia e pode apresentar padrão com bócio difuso ou sem bócio, de acordo com o grupo de citocinas e genes envolvidos (1, 3, 16, 22).

Nos pacientes portadores de TH, os nucleotídeos tireoperoxidase e tireoglobulina, integrantes ativos do processo de síntese hormonal nos tireocitos, são alvos de ataque de autoanticorpos. Altos títulos de anticorpos antitireoperoxidase (AcTPO) são encontrados em 90 a 95% dos pacientes, com presença de anticorpos

antitireoglobulina (AcTG) em 20 a 50% dos pacientes. Eventualmente, encontra-se anticorpos antirreceptor de TSH (TRAb) com efeito bloqueador do estímulo (1, 3, 22).

A reação imune na TH decorre principalmente da resposta imune mediada por células com infiltração de células inflamatórias, causando destruição gradual com consequente dano na função da glândula, manifestando-se por hipotireoidismo clínico ou subclínico associado à atrofia glandular ou bócio, de acordo com o tipo de interleucinas e genes presentes (1, 3, 4, 15 – 17).

Os fatores ambientais funcionam como gatilhos para ativação das reações autoimunes em indivíduos suscetíveis. O contato prévio com radiação está associado a maior prevalência de autoanticorpos além de maior risco para desenvolvimento de disfunções tireoidianas, desenvolvimento de nódulos benignos ou malignos. Concentrações de iodo tanto em excesso quanto em deficiência do mesmo modo associam-se às tireoidopatias autoimunes. Adicionalmente, concentrações adequadas de selênio são necessárias para o controle da resposta imunológica, crescimento celular e funcionamento adequado da tireoide, pois relaciona-se diretamente à ativação das desiodases, enzimas responsáveis pela síntese de hormônios tireoidianos. Tabagismo é citado como fator protetor para TH, pois reduz o risco de ativação de AcTPO e AcTG, além de relatos de presença de receptores nicotínicos em células do sistema imune exercendo inibição de respostas via Th1 e Th17. Porém, tal efeito protetor desaparece em poucos anos após a cessação do tabagismo. Infecções por alguns vírus como parvovírus B-19, vírus C, HIV associam-se ao estímulo de autoimunidade tireoidiana. Drogas imunomoduladoras, interferon-α, amiodarona, exposição a produtos químicos como organoclorados, alguns metais pesados como manganês e cádmio são ainda responsáveis por disfunções tireoidianas. Há relatos de alterações na composição da microbiota associadas ao desenvolvimento das DTA. A ligação da vitamina D com as DTAs vem sendo amplamente estudada em função dos efeitos adicionais anti-inflamatórios e imunomodulatórios desta vitamina confirmados em numerosos estudos que relatam o papel do calcitriol em potencializar a resposta do sistema imune inato e inibir o sistema imune adaptativo (8-10, 24-27).

Muitos estudos demonstraram que células do sistema imune como macrófagos, células dendríticas, linfócitos T e B expressam tanto o receptor de vitamina D quanto 1 alfa hidroxilase (enzima conversora da vitamina D em calcitriol, sua forma ativa). A vitamina D possui efeitos regulatórios diretos na função dos linfócitos T através da inibição da síntese de citocinas Th1 e estimulação da síntese de citocinas Th2 por ação direta em linfócitos TCD4 nativos. Age estimulando a produção de células T regulatórias que por intermédio da síntese de IL-10 bloqueiam desenvolvimento de células Th1 e inibem a secreção de IL-17 pelas células T efetoras. Além disso, o calcitriol exerce efeitos diretos sobre as células B inibindo sua proliferação, diferenciação e síntese de imunoglobulinas (17, 21, 28 - 35).

Adicionalmente, a vitamina D tem efeito inibitório sobre a maturação das células dentríticas impedindo sua diferenciação em monócitos e reduzindo a síntese de citocinas inflamatórias pelas mesmas. Atua regulando a diferenciação de monócitos em macrófagos bloqueando a liberação de citocinas inflamatórias por estas células diminuindo assim a sua capacidade de apresentar antígenos aos linfócitos em função da redução da expressão de moléculas MHC-II em sua superfície. Uma vez que as células dendríticas exercem função crucial no desenvolvimento e manutenção da autotolerância, o papel da deficiência de vitamina D na ação destas células e consequentemente no desenvolvimento de doenças autoimunes tem sido amplamente abordado (27, 29, 30, 36-38).

3.1.2. Diagnóstico

TH apresenta evolução clínica variável desde função tireoidiana normal até hipotireoidismo subclínico e posterior disfunção clínica com elevação dos níveis de TSH associado à queda das concentrações de T4. Nota-se presença marcante de autoanticorpos AcTPO, AcTG e TRAb. Este último em menor frequência e, quando presente, tem efeito bloqueador associando-se ao desenvolvimento da forma sem bócio. A captação tireoidiana de iodo pode variar de diminuída a elevada e não é utilizada para fins diagnósticos (13, 22, 23, 39).

3.1.3. Tratamento

Pacientes que evoluem para hipotireoidismo clínico devem ser tratados com reposição de levotiroxina. Nos casos de hipotireoidismo subclínico, devem ser seguidos os critérios específicos para tratamento.

Estudos mostraram mudanças significativas no padrão de marcadores inflamatórios em pacientes com TH, além de queda nas concentrações de autoanticorpos no decorrer do tratamento (13, 22, 23, 39).

3.2 Doença de Graves

3.2.1 Definição

Doença de Graves (DG) é a causa mais comum de hipertireoidismo em áreas suficientes em iodo com incidência anual de 14 casos a cada 100.000 indivíduos. Sua etiologia é multifatorial, havendo associação com ativação de genes específicos (CD40; FCRL3, PTPN22, CTLA-4), fatores ambientais e imunológicos, estes representados pela alta concentração de citocinas inflamatórias específicas além de perda de imunotolerância associada ao desenvolvimento de autoanticorpos. Além das manifestações tireoidianas, caracteristicamente exibe comprometimento do tecido conjuntivo levando ao desenvolvimento de oftalmopatia quando acomete a região retro-orbitária, mixedema localizado frequentemente pré-tibial e acropatia (40,41).

A presença de autoanticorpos estimuladores das células foliculares através da ligação ao receptor de TSH na célula (TRAb) é definidora na DG. A consequência de sua ação leva à hiperfunção e ao aumento de volume da glândula. Observa-se, pouco menos frequentemente, níveis elevados de outros autoanticorpos como AcTPO e AcTG (3, 40, 41).

A DG decorre principalmente da ativação da imunidade humoral via linfócitos Th2 com presença marcante das citocinas IL-4 e IL-5, produção de TRAb levando à proliferação celular e ao hipertireoidismo. Há estudos evidenciando presença de citocinas dos grupos Th17 e Th22 em altas concentrações no sangue periférico de pacientes com DG que apresentam altos níveis de TRAb. Há também relato de altas

concentrações de IL-17 nos tireócitos de pacientes com DG de difícil controle (1, 12-14, 42, 43).

Dentre os fatores ambientais associados à DG pode-se citar o tabagismo como fator de risco importante tanto para o desenvolvimento de tireotoxicose quanto para oftalmopatia de maior gravidade. Oferta excessiva de iodo pode deflagrar tireoideopatia autoimune. Existem relatos de associação entre DG e estímulos virais, drogas como amiodarona e interferon-α, deficiência de selênio, exposição a produtos químicos, metais pesados e alterações de microbiota. A participação da vitamina D no desenvolvimento da DG vem sendo objeto de estudos, sendo apontada como importante fator na alteração da autotolerância por agir diretamente em células ligadas à resposta autoimune inibindo a proliferação de linfócitos assim como a secreção de citocinas inflamatórias. Em modelos animais, a administração de vitamina D preveniu doenças tireoidianas autoimunes. Em humanos, estudos demonstraram concentrações de vitamina D significativamente mais baixas em hipertireoidismo autoimune comparado ao não autoimune (8,9, 24, 25).

3.2.2. Diagnóstico

A DG caracteriza-se por baixas concentrações de TSH associadas a altas concentrações de T4 livre, T3 livre ou total associados à presença de TRAb. Eventualmente, realiza-se a cintilografia de tireoide com iodo para melhor definição diagnóstica da DG, que evidenciará hipercaptação difusa. A ultrassonografia demonstra bócio difuso associado à hipervascularização da glândula ao *Doppler* (40,41).

3.2.3. Tratamento

Há três formas de tratamento utilizadas para DG: drogas antitireoidianas, radioidoterapia (RIT) e tireoidectomia. As três opções têm sua eficácia comprovada.

Os antitireoidianos, medicamentos da classe das tionamidas, são representados por propiltiouracil e thiamazol. O mecanismo de ação baseia-se na inibição da organificação do iodo por bloqueio da ação da tireoperoxidase no interior da célula tireoidiana. Inibem o acoplamento das iodotirosinas impedindo assim a

formação de T3 e T4. Propiltiouracil em altas doses apresenta a ação adicional de inibição da conversão de T4 em T3 nos tecidos periféricos. Utiliza-se ainda, betabloqueadores para o controle dos sintomas de tireotoxicose (40, 41).

Em situações de falência terapêutica com antitireoidianos após 12 a 18 meses de tratamento podem ser indicados tratamento ablativo como radioiodoterapia (RIT) ou tireoidectomia total (40, 41, 44-46).

Dados epidemiológicos sugerem que a deficiência de vitamina D agiria como um fator de risco para o desenvolvimento de doenças tireoidianas autoimunes. Estudos indicam que o calcitriol exerceria papel modulador tanto no sistema imune inato quanto no adaptativo, isto é, várias linhagens de células de defesa estariam sob efeito e ações da vitamina D. A suplementação de vitamina D com o objetivo de tratamento e até mesmo a prevenção das doenças tireoidianas autoimunes ainda é objeto de muito estudo em seres humanos, uma vez que parece promissor em modelos animais (21, 24, 27, 28, 33, 47 – 57).

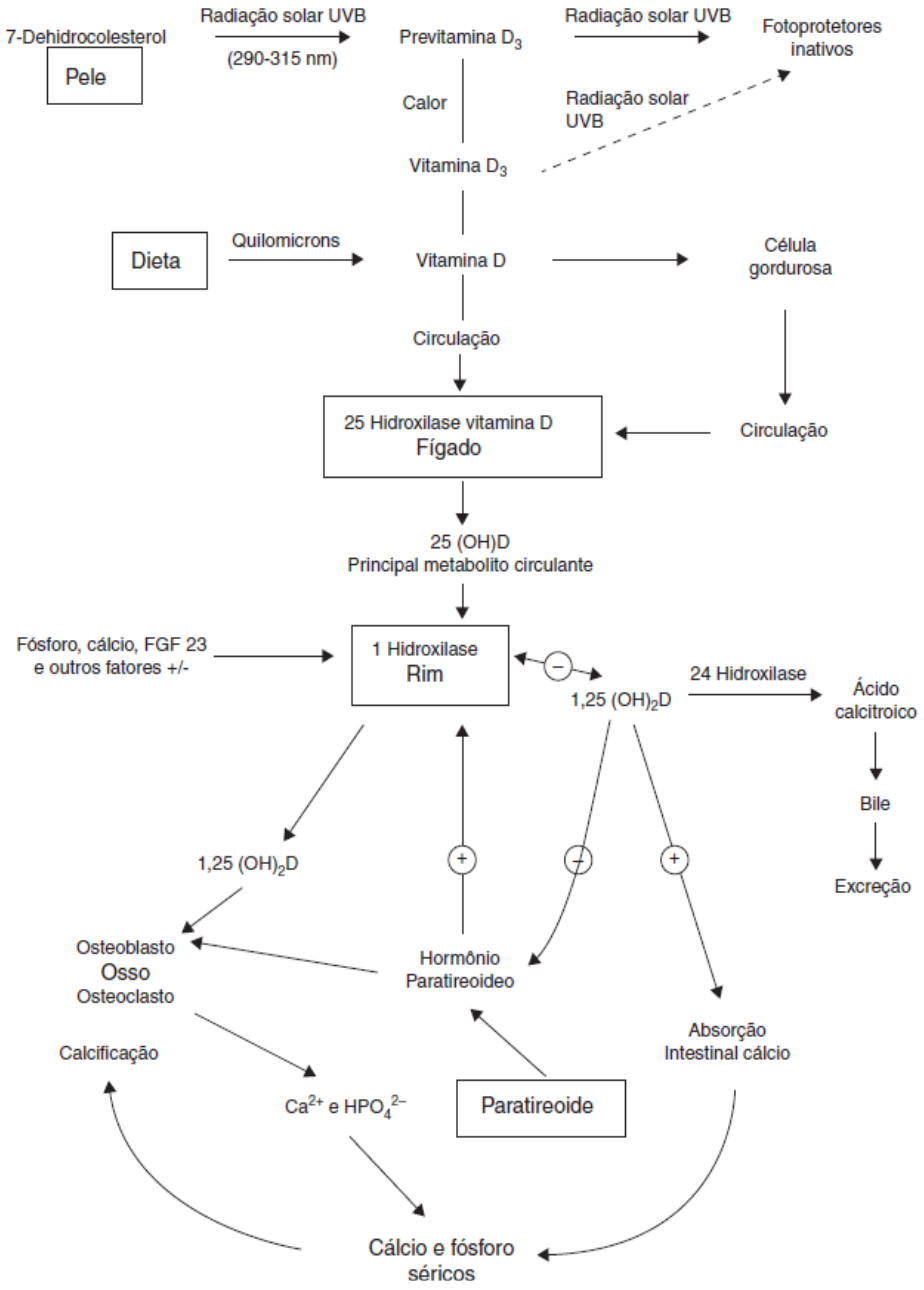
4. Vitamina D

4.1 Sistema Imune e Vitamina D

Grande parcela da população mundial apresenta baixos níveis de vitamina D e a insuficiência da vitamina é apontada como um problema de saúde pública em muitos países. Mais de 50% da população da América do Sul tem níveis de vitamina D abaixo de 20 ng/ml no inverno assim como a população do Ocidente Europeu. Esta porcentagem é menor na América do Norte e maior na África do Sul, ficando entre 20% a 50% na Oceania. Concentrações de vitamina D abaixo de 10 ng/ml são encontradas em maior prevalência no Oriente Médio e em vários países asiáticos como India e China (58-61).

Vitamina D3 (25OHD) ou colecalciferol, embora descrita inicialmente como uma “vitamina”, é considerada um hormônio, conhecida primeiramente por seu papel na regulação da homeostase cálcio-fosfato atuando ativamente na transmissão neuromuscular e na mineralização óssea. A vitamina D é obtida primariamente através da alimentação (vitamina D2 e D3) ou através da exposição da pele à radiação UVB (vitamina D3), sendo esta sua principal fonte. A Vitamina D é

sintetizada na pele como pré-vitamina D tendo como precursor 7-dehidrocolesterol após exposição a raios ultravioleta-B (UVB); em seguida, liga-se à proteína transportadora de vitamina D (DBP), chegando ao fígado, onde sofre a primeira hidroxilação por ação da enzima 25-vitaminaD- 1-α-hidroxilase (mecanismo dependente do citocromo P-450) originando a 25-hidroxi-vitamina D [25(OH)D], principal forma circulante, porém, com baixa atividade biológica. Posteriormente sofre a segunda hidroxilação por ação enzimática (1-α-hidroxilase) em nível renal, sendo então convertida à sua forma ativa, calcitriol ($1,25(OH)_2D$). Os níveis circulantes de calcitriol são regulados por vários fatores. A ação da 1-α-hidroxilase é estimulada por PTH e calcitonina e inibida pelos níveis de cálcio (hipercalcemia) e da própria $1,25(OH)_2D$ (Figura 2) (19, 58 - 64).



25(OH)D: calcidiol 1,25(OH)2D: calcitriol; HPO4: Fosfato; Ca 2+: cálcio;

FGF23: Fator de crescimento de fibroblastos 23;

Figura 1: Síntese e metabolismo da vitamina D.

(Fonte: Urrutia-Pereira M e cols, 2015)

A eficácia da síntese de vitamina D depende de exposição adequada da pele aos raios UVB de forma que a latitude, estação do ano, hora do dia, além da utilização de protetores solares ou até mesmo maior quantidade de melanina na pele interferem nesta síntese. O envelhecimento da pele com a idade diminui a disponibilidade cutânea de 7-dehidrocolesterol. A ingestão reduzida de vitamina D contribui para a queda em seus níveis séricos. São poucos os alimentos que contêm quantidades significativas de vitamina D, dentre os quais podemos citar alguns peixes como bacalhau e salmão fresco, gema de ovo e alguns produtos lácteos enriquecidos com vitamina D. Obesidade igualmente é fator contribuinte para deficiência da vitamina D, pois ficaria retida na gordura corporal uma vez que é lipossolúvel. Doenças que prejudicam absorção de gorduras como Doença Celíaca e Doença de Chron têm seu papel, além de algumas medicações que ativam o citocromo P-450 em nível hepático, aumentando assim o catabolismo da vitamina como anticonvulsivantes, glicocorticoides e antirretrovirais (Figuras 3 e 4) (19, 61-63).

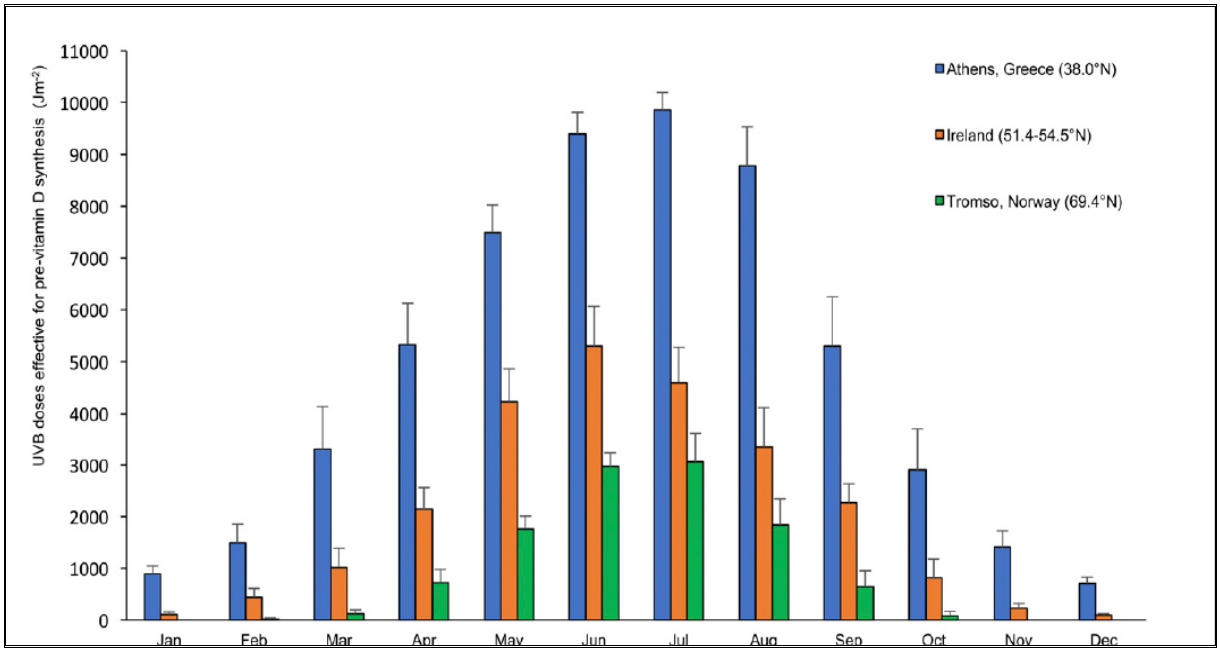


Figura 2: Doses mensais de UVB modeladas eficazes para a síntese de precolecalciferol em Tromsø, Noruega (latitude 698 N), República da Irlanda (latitude 51–548 N) e Atenas, Grécia (latitude 388 N)

(Fonte: O'Neill e cols, 2016)

A enzima 1-α-hidroxilase encontra-se igualmente em várias outras células que expressam receptores de vitamina D como ossos, medula óssea, cérebro, pulmão, músculo, coração e células de defesa. A forma ativa da vitamina D se liga a seu receptor nuclear (VDR), o qual está expresso em várias células do sistema imunológico como monócitos, macrófagos, células dentríticas, linfócitos T e B, promovendo ações imunomodulatórias. Baixas concentrações de vitamina D associaram-se à predisposição a várias doenças autoimunes como diabetes mellitus tipo 1, artrite reumatóide, esclerose múltipla e doenças tireoidianas autoimunes (DTAs). Há relatos da associação entre baixos níveis de vitamina D e presença de anticorpos anti-TPO, assim como a associação a polimorfismos do gene de VDR em pacientes portadores de DTAs (30, 31, 65 - 70).

A expressão de VDR em células do sistema imune e a presença da enzima 25 (OH)D-hidroxilase em células dendríticas e macrófagos sugere que $1,25(OH)_2D_3$ (calcitriol) possui propriedades de regulação hormonal local. A vitamina D ativa (calcitriol) age nas células apresentadoras de antígenos como células dendríticas e macrófagos, inibindo a expresssão de antígenos MHC classe II em sua superfície e impedindo a maturação das células dendríticas com redução da apresentação de antígenos e consequente redução na ativação de linfócitos T (28-32,55).

Calcitriol modula a resposta imune por influenciar a atividade das células T reguladoras, inibindo a produção e ativação de citocinas e a transformação de um estado pró-inflamatório em *status* de imunotolerância decorrente da capacidade da vitamina D em suprimir o sistema imune adaptativo. Ainda, promove diminuição da proliferação de células Th1, Th17 e aumento da concentração de células Th2. A expressão de VDR aumenta cinco vezes em relação à ativação de células T latentes. O aumento de células T reguladoras causado pelo calcitriol *in vitro* e *in vivo*, aparentemente é o responsável pela supressão do processo de autoimunidade. Vitamina D é responsável por inibir a proliferação e a secreção de anticorpos pelas células B, além de induzir apoptose das mesmas (Figura 5) (21,30 - 32, 48, 55).

Os mecanismos fisiopatológicos que envolvem a ação da vitamina D ainda estão longe de serem elucidados. Vários questionamentos foram levantados sobre o papel da mesma na prevenção de doenças crônicas, como doenças autoimunes,

diabetes mellitus tipo 2 e doenças cardiovasculares, porém, ainda são necessários muitos ensaios clínicos para esclarecer seu verdadeiro papel (71-74).

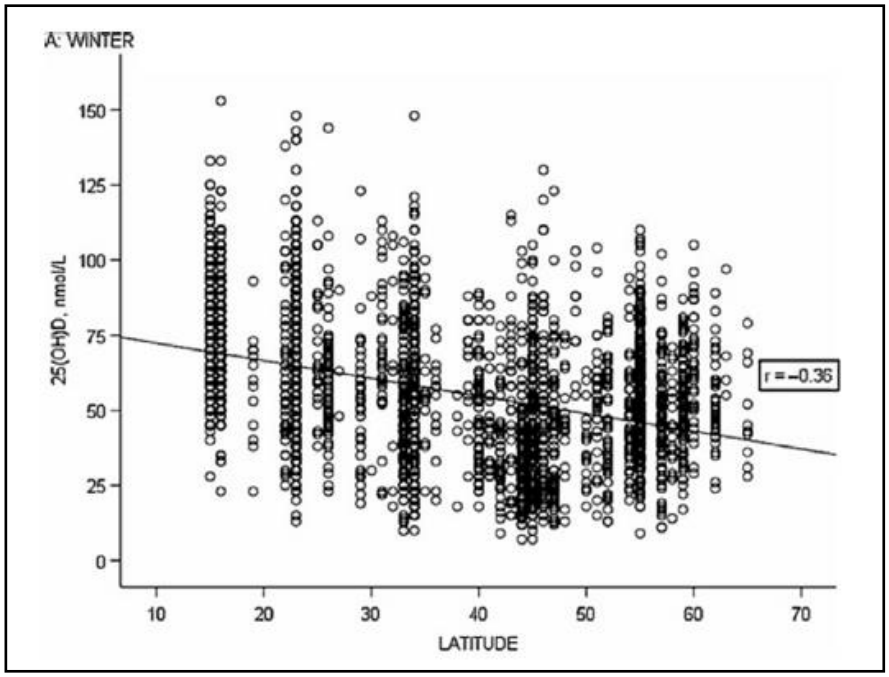
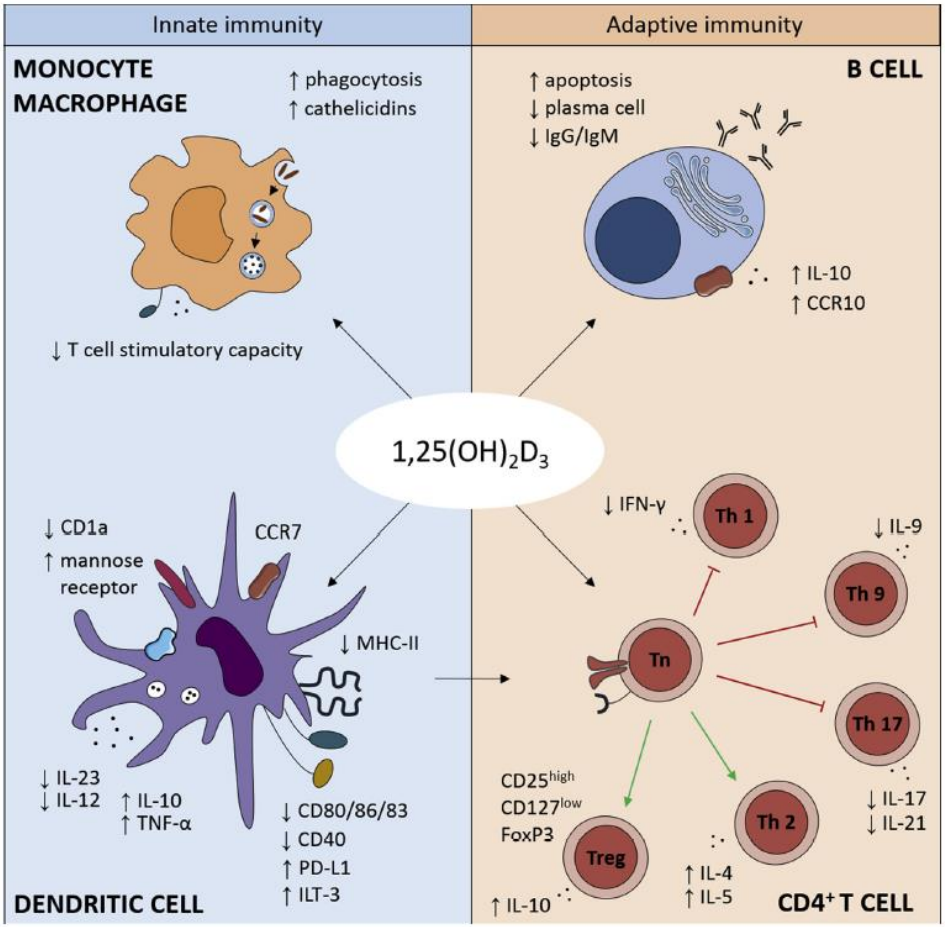


Figura 3: Níveis séricos de vitamina D de acordo com a latitude e no inverno em 3741 mulheres pós-menopausa em 6 continentes.

(Fonte: Kuchuk NO et al. 2009)



IL:interleucina; 1,25(OH)2D3: Calcitriol; CD4+T Cell: linfócito TCD4+; B Cell: Linfócito B; Th: Linfócito T helper; Treg: Linfócito T regulador

Figura 4: Efeitos imunomodulatórios do calcitriol.

(Fonte: Vanherwegen et al. 2017)

4.2 Dosagem sérica de Vitamina D

Concentrações de 25OHD (calcidiol) circulante representam o melhor indicador do *status* de vitamina D (58).

As metodologias de dosagem de vitamina D utilizadas em laboratórios clínicos incluem os ensaios competitivos de proteína de ligação à vitamina D (CPBA), imunoensaios, cromatografia líquida de alta *performance* (HPLC), cromatografia e

espectrometria de massa em *tandem* com líquido (LC-MS/MS). As amostras devem ser cuidadosamente armazenadas em recipientes sob o abrigo da luz (29, 58, 75).

Embora as metodologias de testes de 25OHD tenham se aprimorado significativamente, ainda existe um viés considerável entre os diferentes métodos e laboratórios. Portanto, a padronização de um método faz-se necessária. Os níveis ideais de 25OHD devem ser determinados com base neste método padronizado. Há interferência também quando os ensaios são realizados por diferentes laboratórios. As dosagens de vitamina D são realizadas habitualmente pelas técnicas de imunoensaios, cromatografia líquida e espectometria de massa em tandem (LC-MS/MS), sendo os dois últimos mais precisos nas dosagens. A cromatografia líquida, padrão ouro, também permite diferenciar as concentrações dos metabólitos 25(OH)D2 e 25(OH)D3, porém, a maioria dos estudos tem trabalhado na prática com ensaios quimioluminescentes (CLIA) ou radioimunoensaios (RIA) (29, 58, 75,76).

Um importante fator presente no soro humano e que pode ser responsável pela alteração nas dosagens da 25OHD é o C-3 epímero da vitamina D (3-epi-25(OH)D3). O epímero tem o mesmo peso molecular da vitamina e ambos diferem apenas na estereoquímica do grupo hidroxila na posição 3. Trata-se de um substrato para a enzima 1α- hidroxilase sendo convertido em 3-epi-1,25 dihidroxivitamina D3 e capaz de ligar-se ao receptor de vitamina D, porém sua importância fisiológica ainda permanece incerta (77,78).

As opiniões divergem a respeito de concentrações ideais de vitamina D. De acordo com a *Endocrine Society,* concentrações normais seriam estabelecidas acima de 30 ng/ml. De acordo com o *Instute of Medicine* (IOM), em Washington, que criou um comitê para avaliar os valores de referência baseados em uma revisão atualizada utilizando as referências de consumo dietético, o valor de corte seria 20 ng/ml, de forma que valores abaixo deste nível seriam considerados como insuficiência e valores abaixo de 10 ng/ml como deficiência (58-60).

Em nosso meio, a Sociedade Brasileira de Patologia Clínica/Medicina Laboratorial (SBPC/ML) anunciou a mudança do valor de referência da vitamina D. Segundo nota publicada pelas Sociedades, até então o valor de referência era acima de 30 ng/mL. Porém, desde dezembro de 2017 estão sendo aceitos valores a partir de 20 ng/mL. De acordo com o posicionamento da Sociedade Brasileira de

Endocrinologia e Metabologia (SBEM) lançado no ano de 2017, valores entre 30 e 60 ng/mL são recomendados para grupos de risco como idosos, gestantes, pacientes com osteomalácia, raquitismos, osteoporose, hiperparatireoidismo secundário, doenças inflamatórias, doenças autoimunes, renal crônica e pós-bariátricos (79).

4.3 Doenças Tireoidianas autoimunes e Vitamina D

Baixas concentrações de vitamina D associam-se a maior incidência de doenças autoimunes em geral em função de suas ações imunomodulatórias como a redução da síntese de marcadores inflamatórios. Redução das concentrações de vitamina D ao mesmo tempo associam-se a doenças cardiovasculares, infecções e neoplasias, inclusive a tireoidiana. Vários autores ressaltam o papel da vitamina D no desenvolvimento de autoimunidade tireoidiana, relatando a associação entre polimorfismos no gene de VDR e presença de autoanticorpos tireoidianos. Diferentes graus de redução nas concentrações de vitamina D correlacionam-se com tempo de doença, títulos de autoanticorpos e volume tireoidiano. Sabe-se que o receptor VDR, assim como a enzima 1-α-hidroxilase, estão presentes em várias células do sistema imunológico desde células apresentadoras de antígenos, como as células dendríticas e macrófagos, até linfócitos B e T (21, 48, 55, 70, 73, 80, 81).

As DTAs resultam de predisposição genética associada a influências ambientais e alterações imunológicas caracterizadas por infiltração linfocítica na glândula e produção de autoanticorpos específicos associados ao desequilíbrio entre as respostas imunes Th1 e Th2, resultando em reação autoimune mediada por células via Th1 predominantemente com destruição de tireocitos e hipofunção na TH e em um predomínio de resposta humoral via Th2 produzindo TRAb estimuladores levando ao hipertireoidismo na DG. Ambos os grupos de linfócitos T, além do grupo Th17, têm participação em diferentes graus em ambas as doenças (3, 4, 6, 8, 12-14).

Muitos estudos evidenciaram redução dos títulos de autoanticorpos em pacientes portadores de TH em terapia substitutiva com levotiroxina ou portadores de DG em tratamento com antitireoidianos com concentrações normais de vitamina

D sugerindo que a mesma possa potencializar o tratamento dessas doenças. Contudo, outros autores discordam desta associação. Há relatos de maior taxa de recorrência de DG em pacientes com baixas concentrações de vitamina D no momento em que o tratamento com tionamidas foi descontinuado (21, 54, 82).

A vitamina D poderia agir aprimorando o sistema imunológico inato e regulando o sistema imunológico adaptativo, promovendo tolerância imunológica e contribuindo assim para diminuir a probabilidade de desenvolver doenças auto-imunes. Apesar de vários autores apontarem benefícios no tratamento com vitamina D ainda são necessários grandes estudos prospectivos multicêntricos para que se possa chegar a uma conclusão definitiva liberando sua utilização para tratamento de doenças tireoidianas autoimunes na prática clínica (21, 27, 48, 55, 83, 84).

HIPÓTESE:

Insuficiência de vitamina D está associada a doenças tireoidianas autoimunes, atividade de doença e alterações de marcadores imunológicos.

OBJETIVOS:

1. Geral:

Estudar a prevalência de insuficiência da vitamina D e a relação de sua concentração com perfil hormonal tireoidiano, marcadores séricos de autoimunidade tireoidiana e de atividade inflamatória em pacientes com DG e TH.

2. Específicos:

1. Avaliar a prevalência de insuficiência de vitamina D em pacientes com doença tireoidiana autoimune divididos em 2 grupos: pacientes com DG e pacientes com TH comparados a indivíduos sem doença tireoidiana (grupos de controle);
2. Correlacionar o perfil de atividade inflamatória obtido por intermédio da avaliação de interleucinas Th1, Th2 e Th17 com valores de vitamina D nos 2 grupos de pacientes e no grupo de controle;
3. Avaliar a associação entre concentrações de vitamina D com aspectos laboratoriais da função e autoimunidade tireoidianas, assim como de tratamento nos 2 grupos de pacientes;
4. Avaliar associação entre concentrações de vitamina D e volume tireoideano.

METODOLOGIA:

1. Desenho do estudo:

Estudamos pacientes com TH e DG em relação ao *status* de vitamina D e marcadores de autoimunidade da tireoide, bem como a relação com citocinas produzidas pelas células Th1, Th2 e Th17.

A análise interina foi realizada para avaliar o poder estatístico da correlação entre hormônios tireoidianos, vitamina D e os marcadores de autoimunidade tireoidiana, resultando em um mínimo de 193 pacientes.

Para testar nossa hipótese, desenvolvemos um estudo envolvendo 88 pacientes com TH, 105 pacientes com DG seguidos em hospital universitário e 71 indivíduos sem DTA, com idade entre 18 e 65 anos.

Foram coletadas amostras de sangue dos 3 grupos para dosagem de vitamina D total (25OHD), tireotrofina (TSH), tiroxina livre (T_4L), cálcio, fósforo, paratormônio (PTH), anticorpo antitiroperoxidase (AcTPO), anticorpo antitireoglobulina (AcTG) e antirreceptor de TSH (TRAb). As citocinas produzidas por células Th1 (IL-2, IFN-γ, TNF-α), Th2 (IL-4, IL-5) e Th17 (IL-17) foram dosadas em todos os participantes. O volume de tireoide nos pacientes foi estimado por ultrassonografia. Dados sobre peso, altura, índice de massa corporal, paridade e tempo de diagnóstico dos pacientes foram coletados diretamente por entrevista.

O consentimento pós-informado por escrito foi obtido de cada indivíduo após explicação completa da finalidade e da natureza de todos os procedimentos. O estudo foi aprovado pelo Comitê de Ética em Pesquisa da Universidade (CAAE 03330912.3.0000.5404).

1.1 Critérios de inclusão

Somente pacientes com diagnóstico de TH e DG foram incluídos no estudo.

O diagnóstico de disfunção tireoidiana foi baseado em concentrações de TSH e T4L alteradas. No momento da coleta, todos os pacientes com TH estavam

em terapia de reposição com levotiroxina. Os pacientes com DG foram divididos em dois grupos de acordo com a função tireoidiana. Grupo sem tionamidas: pacientes em eutireoidismo ou hipotireoidismo sob reposição com levotiroxina. Grupo com tionamidas: pacientes em atividade de doença sob tratamento com tionamidas, em eutireoidismo ou hipertireoidismo. Apenas pacientes com altos níveis de anticorpos antitireoidianos confirmando a etiologia de DTA foram incluídos.

Todos os indivíduos do grupo de controle eram clínica e laboratorialmente eutireoideos com concentrações de AcTPO, ACTG e TRAb indetectáveis e foram selecionados entre os acompanhantes dos pacientes e funcionários do hospital.

1.2 Critérios de exclusão

Os critérios de exclusão foram: história prévia de tireoidectomia, pacientes com doenças agudas, doença maligna ou inflamatória ativa, uso de amiodarona, esteróides, cálcio e/ou vitamina D, uso de contraste iodado em período inferior a 3 meses antes do início do estudo, insuficiência cardíaca (classe III ou IV da NYHA), doença hepática grave (albumina reduzida ou INR aumentado), doença renal avançada (estágio 4 ou 5), pacientes em hemodiálise e aqueles soropositivos para HIV e hepatite C, além de outras doenças autoimunes associadas.

2. Avaliação laboratorial do *status* hormonal tireoidiano e de vitamina D

Para avaliar os níveis hormonais de pacientes e grupo de controle, utilizamos as concentrações de TSH medido por eletroquimioluminescência (Roche Cobas Elecsys - valores de referência VR de 0,41 a 4,5 µUI / mL). O coeficiente de variação intra-teste (CV) foi de 5%; faixa de medição 0,01-100 µUI / ml, sensibilidade analítica 0,01 pg / mL e sensibilidade funcional 0,014µUI / ml com CV inter-ensaio de 20%. T4L dosado por imunoensaio competitivo de quimioluminiscência Elecsys FT4 II (VR 0,9 a 1,8 ng / ml). Para o intervalo de T4L foi utilizado entre 0,02 a 7,76 ng / dl, CV intra-teste de 5%, sensibilidade analítica 0,023 ng / ml e funcional 0,39 ng / ml com CV 20% inter-ensaio.

Os anticorpos AcTPO e o AcTg foram medidos pelo teste imunométrico quimioluminescente Elecsys com valores de referência até 34UI/ml para AcTPO e até 115 UI/ml para AcTG. Para o AcTPO, o intervalo de medição foi de 5,0 a 600,0 UI/ml, CV 5%, sensibilidade analítica 5,0 UI / ml e funcional 34 UI/ml. Para o intervalo de medição de ACTG estava entre 10,0 a 4000,0 UI/ml (5% CV); sensibilidade analítica 10 UI/ml e sensibilidade funcional 34 UI/ml. As concentrações de TRAb foram mensuradas por imunoensaio de eletroquimioluminescência competitivo usando receptores de Elecsys TRAb TSH com valores de referência de até 1,22 UI/L; intervalo de medição de 0,3 a 40,0 UI/L, CV 5%; sensibilidade analítica de 0,3 UI/L e sensibilidade funcional de 0,9 UI/L.

A vitamina D total (25OHVitD) foi dosada pelo teste LIAISON® 25 OH Vitamina D TOTAL utilizando a tecnologia de imunoensaio quimioluminescente (CLIA) para a determinação quantitativa de 25-hidroxivitamina D e outros metabolitos hidroxilados de vitamina D em soro humano, plasma ou EDTA com lithium heparin utilizando a avaliação da quantidade de vitamina D com a família dos analisadores LIAISON®. As concentrações de vitamina D abaixo de 30 ng/ml e acima de 20ng/ml foram classificadas como insuficiência, enquanto concentrações acima de 30 ng/ml até 100 ng/ml foram classificadas como vitamina D suficiente. As concentrações de vitamina D abaixo de 10 ng/ml foram classificadas como deficientes, embora alguns autores considerem como deficiência valores de inferiores a 20 ng/ml. Para o presente estudo, valores de vitamina D abaixo de 30 ng/ml foram adotados como insuficiência da vitamina.

3. Avaliação das citocinas

As citocinas foram mensuradas pela técnica de imunoensaio do Milliplex Map com base na tecnologia Lumomex Xmap. Luminex usa técnicas próprias para código de coloração interna de microesferas com dois corantes fluorescentes. Através de concentrações precisas destes corantes, os grânulos de cor distintamente criados estabelecem microesferas de poliestireno ou microesferas magnéticas, cada um dos quais é revestido com um anticorpo de captura específico.

Os valores de referência das citocinas foram: IL-2 VR 1.0-1.6 pg/ml; INF-γ VR 0.8-1.1 pg/ml; TNF-α VR 0.7-1.1 pg/ml; IL-4 VR 4.5-7.1 pg/ml; IL-5 VR 0.5-0.7 pg/ml; IL-17 VR 0.7-1.2 pg/ml.

4. Avaliação ultrassonográfica da tireoide

O volume total tireoidiano foi determinado em mililitros (ml) pelo produto dos diâmetros longitudinal, transversal e anteroposterior multiplicados por uma constante de 0,52; somando-se os volumes dos lobos direito, esquerdo e istmo. Valores entre 6 e 15 ml (10-11 ± 3-4 ml) foram considerados normais para adultos. Os valores foram obtidos de avaliações de rotina que constavam dos prontuários dos pacientes (85).

5. Metodologia estatística

Foi realizada análise exploratória de dados através de medidas resumo (frequência, porcentagem, média, desvio padrão, mínimo, mediana e máximo). Os grupos foram comparados através do teste de Kruskal-Wallis, Qui-Quadrado ou exato de Fisher. A correlação da Vitamina D e Interleucinas com as demais variáveis foi avaliada através do coeficiente de Spearman ou do teste de Mann-Whitney. Os fatores associados à deficiência de Vitamina D foram avaliados através de regressão logística com o critério *stepwise* de seleção de variáveis. O nível de significância estatística para a análise estatística foi 5% (86-88).

PUBLICAÇÕES

Artigo I: Relevancy of thyroid hormone profile and thionamides in status of Vitamin D, inflammatory markers and autoimmunity in Graves' disease

Running head: Vitamin D and thionamides in Graves'disease

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Abstract:

Background/Aims: Graves' disease (GD) may be associated with vitamin D insufficiency and the lower rate of remission after cessation of treatment with thionamides. *Methods*: Cross-sectional study, 105 patients GD, 70 controls. Vitamin D concentrations, thyroid and immunological profile were studied. *Results:* Vitamin D insufficiency was found in 59.1% of the controls, lower than in GD (78.1%, p = 0.028). Comparing GD with (GDT) or without thionamides , IL-4 and TNF-α were lower in GDT. There was higher levels of IL-2 and lower levels of IFN-γ, IL-5, IL-17 in the GD without thionamides when compared to controls. Vitamin D levels did not differ between GDT and without. However, when these two subgroups were compared to controls, lower concentrations of vitamin D were observed in the GDT. There was no difference in levels of FT4, TPOAb, TGAb between GDT or without. *Conclusions*: We demonstrated higher prevalence of vitamin D insufficiency in GD, especially GDT, in relation to the controls, emphasizing the importance of maintaining adequate concentrations of FT4 for the vitamin D status in GD. There is strong evidence for the relationship between GD pathogeny with vitamin D insufficiency. Additional studies are warranted to clarify the role of vitamin D in GD.

INTRODUCTION:

Graves'disease (GD) mainly results from the activation of humoral immunity via Th2 cells with marked presence of IL-4 and IL-5, production of TSH receptor stimulating antibodies (TRAb) leading to cell proliferation and hyperthyroidism. Some studies report the presence of IL-17 and IL-22 in high serum concentrations in patients with GD [1-4].

Several authors highlight the role of vitamin D in triggering thyroid autoimmunity. Its active form (1,25 $(OH)_2VitD_3$) binds to the nuclear vitamin D receptor (VDR), which is expressed in various immune cells such as monocytes, macrophages, dendritic cells, B and T lymphocytes, promoting immunomodulatory actions. There are reports on the association between low vitamin D levels and the presence of anti-TPO antibodies as well as the association of polymorphisms in the VDR gene with ATDs [5-16].

The VDR expression in immune cells and the presence of the enzyme 25(OH)D-1α-hydroxylase in dendritic cells and macrophages suggests that 1,25$(OH)_2$D3 has a local hormonal regulation properties. The active vitamin D modulates the immune response by influencing regulatory T cell activity, inhibiting cytokine production and activation. Consequently, there is decreased proliferation of Th1, Th17 cells and increased concentration of Th2 cells. The increase in regulatory T cells caused by 1,25$(OH)_2D_3$, both *in vitro* and *in vivo* has been suggested as responsible for autoimmunity suppression [7,10,11,13,14,17,18].

Many studies have shown less intense autoimmune reaction and reduction of thyroid autoantibodies in patients with normal levels of vitamin D combining with anti-thyroid drugs. However, other authors not agree with this association. Additionally 1,25$(OH)_2D_3$ acts in the reduction of the synthesis of TRAb by suppression of the B cells, thus contributing to the remission of the GD. There are studies correlating lower concentrations of vitamin D at the time of thionamides discontinuation with the higher recurrence rate of the disease [5,17-20].

Considering the possible role of Vitamin D in thyroid autoimmunity, our aims were to study the relationship of serum vitamin D with thyroid hormone *status,* serum markers of thyroid autoimmunity and inflammation in patients with GD.

MATERIAL AND METHODS:

Study design

We studied GD patients regarding vitamin D status and thyroid autoimmunity markers as well as the relationship with cytokines produced by Th1, Th2 and Th17 cells.

Interim analysis was conducted to evaluate the statistical power of correlation between thyroid hormone, vitamin D and thyroid autoimmunity markers resulting in minimum of 105 patients. We set up a study involving 105 patients with GD followed in our university hospital and 70 individuals without AITD, all aged 18 to 65 years. Blood samples were collected from the 2 groups for measurements of serum total 25OH vitamin D, calcium, phosphorus, parathormone (PTH), thyrotropin (TSH), free thyroxine (FT4), anti-thyroperoxidase antibody (TPOAb), anti-thyroglobulin antibody (TGAb) and anti-TSH receptor antibody (TRAb). Cytokines produced by Th1 cells (IL-2, IFN-γ, TNF-α), Th2 (IL-4, IL-5) and Th17 (IL-17) were measured in all participants.

Thyroid volume in patients was estimated by ultrasound. Data on weight, height, body mass index, parity and time since diagnosis were collected from patients' records or by direct interview.

Written informed consent was obtained from each patient or subject after full explanation of the purpose and nature of all procedures. The University Ethics in Research Committee approved the study (CAAE: 03330912.3.0000.5404).

Inclusion criteria

Only patients with the diagnosis of GD were included in the study. The diagnosis of autoimmune hyperthyroidism was based on low concentrations of TSH and high FT4 with detectable levels of antithyroid antibodies. All subjects in the control group were clinical and laboratory euthyroid, TPOAb, TGAb and TRAb undetectable and were selected from patient's companions or hospital staff.

Exclusion criteria

Exclusion criteria were: previous history of thyroidectomy, acutely ill patients, active malignant or inflammatory disease, use of amiodarone, steroids, calcium and / or vitamin D, use of iodinated contrast less than 3 months before the start of the

study, heart failure (class III or IV NYHA), severe liver disease (reduced albumin or increased INR), advanced kidney disease (stage 4 or 5), patients under hemodialysis and those known to be seropositive for HIV and hepatitis C.

Laboratory evaluation of thyroid and vitamin D status

TSH was measured by eletrochemiluminescence (Roche Cobas Elecsys - reference values: 0.41 to 4.5 µUI / mL) The intra-assay coefficient of variation (CV) was 5%; measuring range 0.01-100 µUI / ml, analytical sensitivity 0.01 pg / mL and functional sensitivity µUI 0,014 / ml with inter-assay CV of 20%. FT4 by competitive chemiluminescence immunoassay Elecsys FT4 II (RV 0,9 to 1,8 ng / ml). For FT4 measurement interval was used between 0.02 to 7.76 ng / dl, intra-assay CVs of 5% analytical sensitivity 0.023 ng / ml and functional 0.39 ng / ml and inter-assay CV 20%.

TPOAb (RV up to 34UI/ml) and TGAb (RV up to 115 IU/ml) were measured by chemiluminescent immunometric assay Elecsys. For TPOAb, measurement interval was 5.0 to 600.0 IU / ml, CV 5% analytical sensitivity 5.0 IU / ml Functional 34 IU / ml. For TGAb measurement interval was between 10.0 to 4000.0 IU / ml (5% CV); analytical sensitivity 10 IU / ml and functional sensitivity 34 IU / ml. TRAb was measured by competitive electrochemiluminescence immunoassay using Elecsys TRAb TSH receptors (RV up to 1.22 IU /L); measurement interval range from 0.3 to 40.0 IU / L, CV 5%; analytical sensitivity of 0.3 IU / L and functional sensitivity of 0.9 IU / L.

Total vitamin D (25OHVitD) was measured by The test LIAISON® 25 OH Vitamin D TOTAL using chemiluminescent immunoassay technology (CLIA) for the quantitative determination of 25-hydroxyvitamin D and other hydroxylated metabolites of vitamin D in human serum, plasma or EDTA plasma with lithium heparin using the evaluation of the amount of vitamin D using the family LIAISON® analyzers. Vitamin D concentrations below 30 ng / ml and above 20ng/ml were classified as insufficiency, while concentrations above 30 ng/ml until 100 ng/ml were classified as vitamin D sufficient. Vitamin D concentrations below 10 ng/ml were classified as deficient although some studies consider as deficiency values below 20 ng/ml.

Cytokines evaluation

Cytokines were measured by the immunoassay technique of Milliplex Map based on the Luminex Xmap technology. Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets polystyrene microspheres or magnetic microspheres can be created, each of which is coated with a specific capture antibody. RV: IL-2 : 1.0-1.6 pg/ml; INF-γ : 0.8-1.1 pg/ml; TNF-α : 0.7-1.1 pg/ml; IL-4 : 4.5-7.1 pg/ml; IL-5 : 0.5-0.7 pg/ml; IL-17 : 0.7-1.2 pg/ml.

Thyroid ultrasound evaluation

The total thyroid volume was determined in milliliters (ml) by the product of the longitudinal, transverse and anteroposterior measurements multiplied by the constant 0.52, adding up the volumes of the right and left lobes and isthmus. Values between 6 and 15 ml (10-11 ± 3-4 ml) were considered normal for adults [21].

Statistical Methods:

Exploratory data analysis was performed through summary measures (frequency, percentage, mean, standard deviation, minimum, median and maximum). The groups were compared using Kruskal-Wallis, Qui-Square or Fisher's exact test. The correlation of Vitamin D and Interleukins with the other variables was evaluated using the Spearman coefficient or the Mann-Whitney test. Factors associated with vitamin D deficiency were assessed through logistic regression using the *stepwise* selection criteria. The significance level for statistical analysis was 5% [22-24].

RESULTS:

The study included 175 participants, 105 patients with GD, of which 87 were female (82%) and 70 subjects in the control group, 61 female (85.9%). Mean time of diagnosis was 8 years (range 1-33 years). Among the patients, 25 (23.8%) were in remission (without medication), 29 (27.6%) were on thionamide and 51 (48.5%) were on levothyroxine treatment. Of the 105 patients, 83 were in euthyroidism and 4 in clinical hyperthyroidism. All subjects in the control group were euthyroid.

Vitamin D insufficiency was found in 59.1% (n=39) of the individuals in the control group and in 78.1% of GD (n=82; p = 0.0078). GD had lower vitamin D and phosphorus and higher PTH when compared to control group (Table 1). We observed that GD had higher IL-2 (Th1) and lower TNF-α (Th1), IFN-γ (Th1), IL-5 (Th2) and IL-17 (Th17) compared to control group (Table 2).

When comparing GD with or without thionamides treatment, IL-4 and TNF-α were lower in GD using thionamides as well as with higher thyroid volumes and lower TSH in relation to the patients without thionamides (Table 3).

There was no difference in IL-4 between GD under thionamides and control group, while TNF-α was lower in the thionamide group. IL-2 was higher in the non-thionamide group when compared to controls, whereas IFN-γ, IL-5 and IL-17 were higher in controls (Table 4).

Vitamin D concentrations did not differ between patients with and without thionamides. However, when these two subgroups were compared to controls, lower concentrations of vitamin D were observed in the DG subgroup using thionamides (table 3; table 4).

In the comparison between the patients with GD under thionamides and non-thionamides, there was no difference in the concentrations of FT4, TPOAb, TGAb, PTH and IL-4.

Correlations*:*

We observed lower concentrations of vitamin D in females than in males in GD group [p = 0.0339, mean 23.95 ng / ml (10.3-42.5) vs. 28.78 ng / ml (9.6-60)].

As shown in table 5, in GD there was an inverse correlation between vitamin D with calcium and thyroid volume and not with immunological markers, phosphorus, PTH or thyroid hormone profile.

We observed an inverse correlation between IFN-γ and IL-5 with time of diagnosis, besides IL-4 with free T4. IL-4 was still positively correlated with phosphorus. No correlation was observed between other interleukins and calcium, TSH, TPOAb , TGAb, TRAb, PTH, or thyroid volume (Table 5).

In the control group, there was a negative correlation between vitamin D and age (r = -0.31374, p = 0.013) whereas there was no correlation between vitamin D and

interleukins, thyroid hormone profile, calcium, phosphorus or PTH . We also did not observe correlation between interleukins and calcium, phosphorus, PTH, TSH or free T4 (data not shown).

Analysis of Logistic Regression:

After simple logistic regression analysis, it was verified that in GD, calcium (OR = 3.136; p = 0.0337 CL95%: 1.092-9.009) was a predictor of vitamin D insufficiency. There were no significant values between vitamin D insufficiency and the other variables (data not shown).

In the multiple logistic regression analysis, free T4 (OR = 0.027, 95% CI: 0.00.0216) and IFN-γ (OR = 0.889, 95% CI: 0.803-0.985, p = 0.0245) were found as predictors for vitamin D insufficiency in GD.

A receiver operator characteristic (ROC) curve was built to discriminate the best threshold for concentrations of free T4 to discriminate insufficiency and sufficiency of vitamin D. We found a threshold of 1.365 *ng/dl*, with an accuracy of 64.5%, specificity of 52.2% and sensitivity of 76.8% (figure 1).

In the control group, age (OR = 1.051, p = 0.0182, CL95%: 1.008 - 1.095) and IL-4 (OR = 1.013, p = 0.0415, CL95%: 1.000 – 1.026) was predictive factor of vitamin D insufficiency. There were no significant values between vitamin D insufficiency and the other variables: sex, calcium, phosphorus, PTH, TSH, free T4 and other interleukins studied.

DISCUSSION:

The present study demonstrated that vitamin D insufficiency was more prevalent in patients with GD and more pronounced in GD under use of thionamides than in the control group. Ma et al (2015) verified the association between lower vitamin D concentrations and AITDs (GD, Hashimoto's thyroiditis and postpartum thyroiditis) when compared to controls, similarly Wang (2015) and Mazokopakis (2014). In this sense, Ahn et al. (2017) found that the lower vitamin D concentrations at the time of discontinuation of thionamides, the major was the rate of recurrence of GD in these patients and showed lower concentrations of vitamin D and higher concentrations of FT4 as predictors of disease. Yasuda et al (2013) reported

significantly lower vitamin D concentrations in patients without GD remission compared to remission patients [5,9,10,17,25,26].

We emphasize that free T4 was a predictor of vitamin D insufficiency for Graves' disease, especially at concentrations below 1.365 ng/dl, but not for the euthyroid individuals of the control group, suggesting that the thyroid hormone status would play a fundamental role in the maintenance of vitamin D sufficiency and its immunomodulatory role would influence the presence of thyroid autoimmune disease. Ahn et al. (2017) showed a significant association between lower concentrations of vitamin D and lower disease remission rate after discontinuation of the medication. On the other hand, Yasuda et al. (2012), Ma et al. (2015), Zhang et al. (2015), Ke et al. (2017) and Planck et al. (2018) found no association between free T4 and TSH with vitamin D insufficiency in GD [10, 17, 20, 27-30].

In this study, IFN-γ was a predictor of vitamin D insufficiency in GD, and is of crucial importance for the onset of autoimmune thyroid disease and closely related to vitamin D concentrations (Mazokopakis et al 2014, Yang Et al 2013, Cantorna et al 2010, Wang et al 2015, Phenekos et al 2004, Yasuda et al 2012). Particularly, we found an inverse correlation between higher concentrations of IFN-γ and IL5 in subjects with a shorter diagnosis time of GD, suggesting that these cytokines, associating with vitamin D insufficiency, would play a relevant role in the initial phase of autoimmune disease [5,11,14,26,30,31].

In addition, in GD, IL-4 was inversely correlated with free T4. The treatment with thionamides, performed in part of our patients resulted in median free T4 normal, however associated a higher frequency of vitamin D insufficiency. In this way, patients with an initial diagnosis of GD or with decompensated hyperthyroidism present higher IL-4, suggesting a possible influence of the thionamides and/or Vitamin D state on this interleukin, therefore the treatment of the disease with antithyroid drugs may interfere with the expression of cytokines. On the other hand there are studies demonstrating that there is no association between interleukins and vitamin D in patients with GD [5,17,18,29,31].

It was still observed a more pronounced decrease in vitamin D with patients presenting higher thyroid volumes, generally associated to lower rates of hyperthyroidism remission, similarly to Bizzaro et al. (2015), who reported in patients

with AITDs the relation between low vitamin D, the presence of anti- thyroid antibodies, higher thyroid volumes and higher levels of TSH. They described as a predisposing factor the VDR polymorphism present in cells of the immune system. Besides that, Pani et al. found polymorphisms in the vitamin D carrier protein (DBP) in GD. In this way, it is suggested the interference of vitamin D insufficiency in the goiter growth in GD possibly due to the increase in the production of stimulating TRAb by B lymphocytes, activated in these states of vitamin D insufficiency [16,32].

Immunological markers of autoimmune thyroid disease in GD, as TRAb, TPOAb and TgAb, showed no significant correlation with vitamin D concentrations as reported by Goswami et al, Effraimids et al and Zhang et al. On the other hand, Choi et al showed a strong association between hypovitaminosis D and TPOAb [9,19, 20,33].

Calcemia was also evidenced as a predictor of vitamin D insufficiency in GD, confirming the influence of the classic calcium-vitamin D interrelationship. In addition, resolution of the hyperthyroidism frequently courses with prolonged hypocalcemia attributed to the transient increase in bone formation due to loss during the thyrotoxicosis state [34-39].

Interestingly, in the control subjects, there was no correlation between vitamin D and interleukins concentrations or thyroid hormone profile, only correlating inversely with age, a finding corroborated by other authors [17,19,25,28, 29].

Patients with GD presented lower serum levels of vitamin D comparing with euthyroid individuals in control group, finding in line with previous works, but interestingly, markedly when in use of thionamides, that is, patients with active disease. This point could reflect the existing autoimmune changes in active Graves' disease. On the other hand, we found no difference when comparing patients on thionamide to untreated patients, perhaps due to the characteristic decrease in vitamin D concentrations associated with the autoimmune process or even insufficient time to recover their stocks after a period under hyperthyroidism. Furthermore, the influence of environmental factors, such as insufficient sunshine exposure or the presence of VDR polymorphisms could also contribute to lower vitamin D concentrations. Conversely, vitamin D has been associated with higher risk of development of autoimmunity and implicated in prevention of autoimmune

diseases. Therefore, some authors consider a future possibility of treating vitamin D deficiency to prevent autoimmune diseases but available data remain controversial [9,13,25,26,40,41].

We considered as limitations of this study the small number of patients and individuals in the control group, nevertheless allowed to obtain robust results. Blood samples were collected in spring and summer, however in our region the seasons do not show substantial differences for sun offered during the months.

In conclusion, our study demonstrated a higher prevalence of vitamin D insufficiency in patients with GD especially under treatment with thionamides, in relation to the control group without thyroid disease, suggesting a possible link between the autoimmunity state or interference of the drug during the active phase of the disease. Furthermore, the presence of lower thyroxine levels in GD, even though within the reference values, as a predictor factor of vitamin D insufficiency, perhaps this reflects that reaching adequate levels of thyroxine facilitate the maintenance of recommended levels of vitamin D, a fact not verified in euthyroid individuals of control group. In addition, there was evidence for the relationship between Graves' disease pathogeny with vitamin D insufficiency.

Additional studies are warranted to clarify the precise role of vitamin D in GD.

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CONFLICT OF INTEREST:

None of the authors has any potential conflicts of interest associated with this research.

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Table 1: Demographic and laboratory characteristics of GD and control group [mean (min-max)*]

Characteristics	**Patients GD (n = 105)**	**Control Group (n=71)**	**P**
Age (years) *	46.3 (20 -69)	47.4 (19 – 77)	0.5131
Gender (F/M) (**n**)	87/18	61/09	0.4419
BMI (Kg/m^2)*	27.1 (17-38)	26.4(17–42.6)	0.2789
Time of diagnosis (years)*	9.6 (1-33)	----------	---------
FT4 (ng/dl)*	1.4 (0.4-7.8)	1.2 (0.8-1.6)	0.1574
TSH (µUI/ml)*	3.1 (0.0-39)	2.0 (0.6-5.0)	0.6903
TPOAb (UI/ml)*	462.5 (30-3000)	----------	---------
TGAb (UI/ml)*	407.7(100-4000)	----------	---------
TRAb (UI/L)*	7.9 (0.2-40)	----------	---------
Calcium (mg/dl)*	9.1(6.9-10)	9.1(8.5-10.1)	0.64
Phosphor (mg/dl)*	3.1(2-4.2)	3.4(2.5-4.5)	0.0002
PTH (pg/ml)*	43.1 (13-98.8)	38.4 (11-127.1)	0.0379
25OHVitaminD (ng/ml)*	24.8 (9.6-60)	28.6 (13-51.2)	0.0071
Vitamin D Insufficiency **n(%)**	82 (78.1%)	39 (59.1%)	0.0078

Dunn Multiple Comparison

Phosphor: Graves < Control; VITD: Graves < Control; PTH: Graves > Control

Table 2: Serum concentrations of interleukins Th1 (IL-2, TNF-α, IFN-γ), Th2 (IL-4, IL-5) and Th17 (IL-17) in GD and control group [mean (min-max)]

Interleukins	GD (n = 105)	Control Group (n=70)	P
IL-2 (pg/ml)	0.7(0.5-8.7)	0.6 (0.1-5.7)	< 0.0001
IL-4 (pg/ml)	34.5 (0.1-213.8)	48.0 (0.9-378.3)	0.1890
IL-5 (pg/ml)	0.6 (0-14.3)	1.6(0-20.8)	<0.0001
TNF-α (pg/ml)	7.8 (0.2-39.9)	11.8 (0.2-35.5)	< 0.0001
IFN-γ (pg/ml)	3.9 (0-47.6)	13.4(0.1-152)	< 0.0001
IL-17 (pg/ml)	3.6 (0.2-54.8)	12.3(0.1-177)	< 0.0001

Dunn Multiple Comparison

IL2: Graves > Control; IFNg: Graves < Control; TNFa: Graves < Control; IL5: Graves < Control; IL17: Graves < Control

Table 3: Comparative analysis of GD with and without thionamides (Mean ± SD)

Variable	GD Without Thionamides (n=76)	GD Thionamides Therapy (n=29)	P
IL – 4 (pg/ml)	51.7 ± 102.0	27.1 ± 40.3	0.0235
TNF – α (pg/ml)	9.4 ± 13.3	7.4 ± 8.7	0.0315
Volume (ml)	11.0 ± 13.1	24.4 ± 12.3	< 0.0001
TSH (µUI/ml)	3.7 ± 6.2	1.3 ± 1.4	0.0001
Vitamin D (ng/ml)	25.4 ± 8.0	22.9 ± 6.4	0.1053
Vitamin D Insufficiency (n%)	56 (73.7%)	25 (89.3%)	0.089

Table 4: Comparative analysis of GD with and without thionamides and control group (Mean ± SD)

Variable	Control group n = 70	GD Without Thionamides n = 76	GD Thionamides Therapy n = 29	P
Time of Diagnosis (years)	----------	11.4 ± 6.7	4.9 ± 3.2	< 0.0001
Thyroid Volume (ml)	----------	11.0 ± 13.1	24.4 ± 12.3	< 0.0001
TSH (µUI/ml)	2.0 ± 1.1	3.7 ± 6.2	1.3 ± 1.4	0.0001
FT4 (ng/dl)	1.2 ±0.2	1.3 ± 0.8	1.6 ± 1.4	0.3074
TRAb (UI/L)	----------	5.9 ± 9.6	11.6 ± 12.9	0.0473
Vitamin D (ng/ml)	28.6 ± 9,2	25.4 ± 8.0	22.9 ± 6.4	0.0081
VitaminD Insuficiency (n%)	39 (59.1%)	56 (73.7%)	25 (89.3%)	0.0097
IL-2 (pg/ml)	0.6 ± 0.8	0.7 ± 1.0	0.6 ± 0.4	< 0.0001
IFN-γ (pg/ml)	13.4 ± 29.0	4.1 ± 9.8	3.4 ± 7.6	< 0.0001
IL-5 (pg/ml)	1.6 ± 3.5	0.5 ± 2.0	0.6 ± 1.7	< 0.0001
IL-17 (pg/ml)	12.3 ± 24.4	3.7 ± 8.8	3.4 ± 4.7	< 0.0001
TNFα (pg/ml)	11.8 ± 6.1	8.0 ± 5.4	7.4 ± 8.7	< 0.0001
IL-4 (pg/ml)	48 ± 76.2	37.1 ± 42.1	27.1 ± 40.3	0.1173

IL2: Graves without thionamides > Control;
TNFa: Graves with thionamides < Control;
IFNg, IL5, IL17: Graves with and without thionamides < Control;
VitD: Graves with thionamides < Control;
TSH: Graves with thionamides < Control, GD without

Table 5: Correlations between interleukins, vitamin D and variables in Graves' disease group.

	Age	Time of diagnosis	Phosphor	FT4	Calcium	Thyroid volume
IFN- γ **r** **p**	NC	- 0.23221 0.0177	NC	NC	NC	NC
IL-4 **r** **p**	NC	NC	0.22906 0.0206	- 0.2183 4 0.0267	NC	NC
IL-5 **r** **p**	- 0.19529 0.047	- 0.26193 0.0072	NC	NC	NC	NC
Vitamin D **r** **p**	NC	NC	NC	NC	- 0.23706 0.0154	- 0.23549 0.0156

Correlation coefficients (Spearman 's rho)

a) **NC:** There was no correlation

b) **r:** correlation

c) **p:** significance statistical level

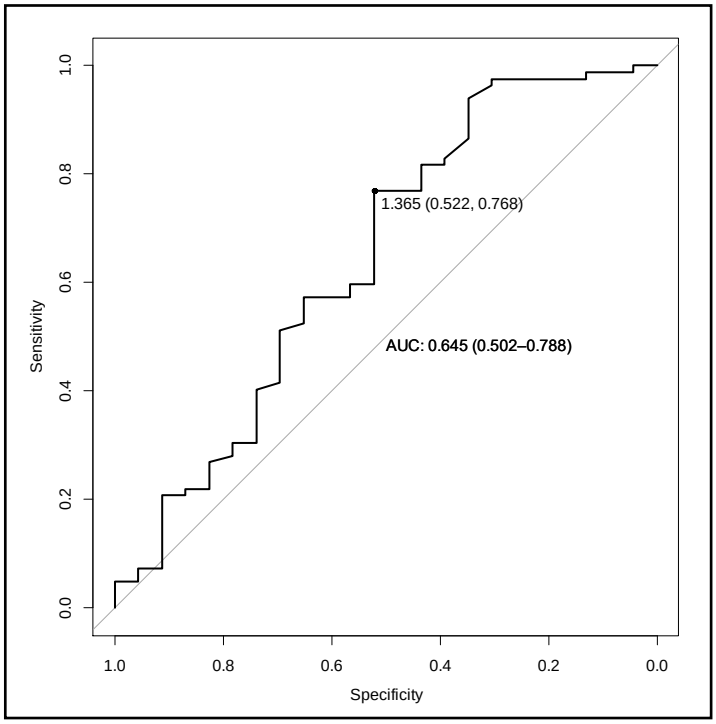

Figure 1: ROC (receiver operator characteristic) curve used to identify threshold values of free T4 related to insufficiency of 25OHvitamin D (< 30 ng/ml) in patients with Graves' Disease. For a concentration of free thyroxine of 1.365 ng/dl, sensitivity was 76.8%, specificity was 52.2%, and accuracy was 64.5%.

Artigo II: Vitamin D in Hashimoto's thyroiditis and its relationship with thyroid function and inflammatory status.

Running head: Vitamin D and cytokines in thyroiditis

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ABSTRACT:

Several studies have shown the correlation between vitamin D [25(OH)D] deficiency and thyroid autoimmunity and reducing of thyroid autoantibodies in patients with normal levels of vitamin D combining with thyroid hormone replacement. However, other authors not agree with this association. It is still unclear whether the low 25(OH)D levels are the result of Hashimoto Thyroiditis (HT) disease or a part of its cause. We studied 88 patients with HT regarding vitamin D status and thyroid autoimmunity markers as well as the relationship with cytokines produced by Th1, Th2 and Th17 cells comparing with a control group of 71 euthyroid healthy subjects. The present study demonstrated that vitamin D concentrations were similar in patients HT and the control group. The reduction of free T4 levels was a predictor of vitamin D insufficiency for Hashimoto's thyroiditis, but not for the control group. Lower concentrations of TNF-α was a predictor of lower levels of vitamin D. Differences in the association between HT and vitamin D insufficiency remain unresolved in the literature. The thyroid hormone status would play a role in the maintenance of vitamin D sufficiency and its immunomodulatory role would influence the presence of thyroid autoimmune disease. The positive correlation between free T4 and vitamin D concentrations suggests that adequate levothyroxine replacement in HT would be an important factor in maintaining vitamin D at sufficient levels.

Keywords: Hashimoto’s thyroiditis, Cytokines, Vitamin D, Autoimmunity

INTRODUCTION:

Hashimoto's Thyroiditis (HT) is one of the autoimmune thyroid diseases (AITDs), also called chronic lymphocytic thyroiditis. Autoimmune attack on the thyroid plays with infiltration of the gland by T and B lymphocytes associated with thyroid peroxidase antibodies (TPO-Ab) and thyroglobulin antibodies (TG-Ab) production [1-5].

In HT occurs a genetic defect in T cell suppressor (Treg) function and CD4+ T cells are not deleted when they are free to promote activation of B-lymphocytes. Concomitantly, the Th cells produce cytokines that induce thyrocytes to express surface antigens HLA-DR making them susceptible to immune attack. There is an interaction between susceptibility genes and environmental factors associated with autoimmune dysfunction [1-3,6].

Th1 cells secrete inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor alpha (TNF-α), essential for cell-mediated immune response. Th2 cells secrete the inflammatory cytokines IL-4 and IL-5, important for the antibody-mediated immune response. Th-17, another Th cell subtype composed of CD4 + T lymphocytes shows involvement in the pathophysiology of autoimmune diseases, especially in HT, and the production of interleukins such as IL-17 and IL-23 [2,7-9].

TH manifests clinically in goiter or non-goiter (or atrophic) forms. The goiter form is related to the predominance of cellular immunity through activation of Th1 promoting apoptosis of the thyroid follicular cells leading to the dysfunction. The non-goiter form is related to the predominance of humoral immunity, via activation of Th2 that induces antigen-specific B lymphocytes to produce anti-TSH receptor antibodies (TRAb) stimulus blockers thus causing the disease [3,10].

Several studies have shown the correlation between vitamin D deficiency and thyroid autoimmunity. Vitamin D3 (cholecalciferol) is produced in the skin when in the presence of ultraviolet-B radiation (UVB) and can also be obtained through supplementation and diet. There is an hydroxylation in the liver and an action of 1-alpha-hydroxylase in the kidney for product its active form calcitriol (1,25 $(OH)_2$VitD), that binds to the nuclear vitamin D receptor (VDR), which is expressed in various immune cells such as monocytes, macrophages, dendritic cells, B and T

lymphocytes, promoting immunomodulatory actions. Low concentrations of vitamin D have been associated with predisposition to various autoimmune diseases such as type 1 diabetes mellitus, rheumatoid arthritis, multiple sclerosis and autoimmune thyroid diseases (AITDs). There are reports on the association between low vitamin D levels and the presence of TPO-Ab as well as the association of polymorphisms in the VDR gene in patients with AITDs. Calcitriol inhibits proliferation of Th1 cells and production of cytokines as well as induces B cell apoptosis [3,11-21].

Many studies have shown reducing of thyroid autoantibodies in patients with normal levels of vitamin D combining with thyroid hormone replacement. However, other authors not agree with this association. It is still unclear whether the low 25(OH)D levels are the result of HT disease or a part of its cause [22-24].

Considering the possible role of Vitamin D in thyroid autoimmunity, our aims were to study the relationship of vitamin D with thyroid function and inflammatory *status* in patients with HT.

METHODS:

Study design

We studied patients with HT regarding vitamin D status and thyroid autoimmunity markers as well as the relationship with cytokines produced by Th1, Th2 and Th17 cells comparing with a control group of euthyroid healthy subjects.

Interim analysis was conducted to evaluate the statistical power of correlation between thyroid hormone, vitamin D and thyroid autoimmunity markers resulting in minimum of 88 patients. Then, we included 88 patients with HT followed in our university hospital and 71 individuals without AITD, all aged 18 to 65 years. Blood samples were collected from the 2 groups for measurements of serum total 25OH vitamin D, thyrotropin (TSH), free thyroxine (FT4), calcium, phosphorus, parathormone (PTH), TPOAb, TGAb and TRAb. Cytokines produced by Th1 cells (IL-2, IFN-γ, TNF-α), Th2 (IL-4, IL-5) and Th17 (IL-17) were measured in all participants.

Thyroid volume in patients was estimated by ultrasound. Data on weight, height, body mass index, parity and time since diagnosis were collected by direct interview. Written informed consent was obtained from each patient or subject after

full explanation of the purpose and nature of all procedures. The study was approved by the University Ethics in Research Committee.

Inclusion criteria

Only patients with the diagnosis of HT were included in the study.

The diagnosis of thyroid dysfunction was based on high concentrations of TSH and low FT4. At the time of collection, all patients with HT were on replacement therapy with levothyroxine. Only patients with high levels of antithyroid antibodies confirming the etiology of AITD were included.

All subjects in the control group were clinical and laboratory euthyroid, TPOAb, TGAb and TRAb undetectable and were selected from patient's companions or hospital staff.

Exclusion criteria

Exclusion criteria were: previous history of thyroidectomy, acutely ill patients, active malignant or inflammatory disease, use of amiodarone, steroids, calcium and / or vitamin D, use of iodinated contrast less than 3 months before the start of the study, heart failure (class III or IV NYHA), severe liver disease (reduced albumin or increased INR), advanced kidney disease (stage 4 or 5), patients under hemodialysis, seropositive for HIV or hepatitis C, and pregnants.

Laboratory evaluation of thyroid and vitamin D status

For evaluation of thyroid status TSH was measured by eletrochemiluminescence (Roche Cobas Elecsys - reference values RV 0.41 to 4.5 µUI / mL). The intra-assay coefficient of variation (CV) was 5%; measuring range 0.01-100 µUI / ml, analytical sensitivity 0.01 pg / mL and functional sensitivity µUI 0,014 / ml with inter-assay CV of 20%. FT4 dosed by competitive chemiluminescence immunoassay Elecsys FT4 II (RV 0,9 and 1,8 ng / ml). For FT4 measurement interval was used between 0.02 to 7.76 ng / dl, intra-assay CVs of 5% analytical sensitivity 0.023 ng / ml and functional 0.39 ng / ml and inter-assay CV 20%. TPOAb and TGAb were measured by chemiluminescent immunometric assay Elecsys (RV up to 34UI/ml and up to 115 IU / Ml, respectively). For TPOAb, measurement interval was

5.0 to 600.0 IU / ml, CV 5% analytical sensitivity 5.0 IU / ml Functional 34 IU / ml. For TGAb measurement interval was between 10.0 to 4000.0 IU / ml (5% CV); analytical sensitivity 10 IU / ml and functional sensitivity 34 IU / ml. TRAb was measured by competitive electrochemiluminescence immunoassay using Elecsys TRAb TSH receptors (RV up to 1.22 IU/L); measurement interval range from 0.3 to 40.0 IU / L, CV 5%; analytical sensitivity of 0.3 IU / L and functional sensitivity of 0.9 IU / L.

Total vitamin D (25OHVitD) was measured by the test LIAISON® 25 OH Vitamin D TOTAL using chemiluminescent immunoassay technology (CLIA) for the quantitative determination of 25-hydroxyvitamin D and other hydroxylated metabolites of vitamin D in human serum, plasma or EDTA plasma with lithium heparin using the evaluation of the amount of vitamin D using the family LIAISON® analyzers. Vitamin D levels between 30 and 60 ng / mL is recommended for at-risk groups as patients with autoimmune diseases and was adopted as adequate to the study population.

Cytokines evaluation

Cytokines were measured by the immunoassay technique of Milliplex Map based on the Luminex Xmap technology. Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets polystyrene microspheres or magnetic microspheres can be created, each of which is coated with a specific capture antibody. The reference values of cytokines are described below: IL-2 RV 1.0-1.6 pg/ml; INF-γ RV 0.8-1.1 pg/ml; TNF-α RV 0.7-1.1 pg/ml; IL-4 RV 4.5-7.1 pg/ml; IL-5 RV 0.5-0.7 pg/ml; IL-17 RV 0.7-1.2 pg/ml.

Thyroid ultrasound evaluation

The total thyroid volume was determined in milliliters (ml) by the product of the longitudinal, transverse and anteroposterior measurements multiplied by the constant 0.52, adding up the volumes of the right and left lobes and isthmus. Values between 6 and 15 ml (10-11 ± 3-4 ml) were considered normal for adults [25].

Statistical Methods:

Exploratory data analysis was performed through summary measures (frequency, percentage, mean, standard deviation, minimum, median and maximum). The groups were compared using Kruskal-Wallis, Qui-Square or Fisher's exact test. The correlation of Vitamin D and Interleukins with the other variables was evaluated using the Spearman coefficient or the Mann-Whitney test. Factors associated with vitamin D deficiency were assessed through logistic regression using the *stepwise* selection criteria. The significance level for statistical analysis was 5% [26-28].

RESULTS:

Descriptive analysis:

The study included 159 participants, 88 patients with HT, of which 82 were female (93 %). In the control group, there were 71 subjects, 61 female (85.9%). Mean time of diagnosis of HT was 10 years (range 1- 47 years). Clinical and laboratorial characteristics of patients and control groups are described in table 1.

Vitamin D levels below 30 ng/dl were found in 59.1% (n=39) of the control group and in 71.8% (n=61) of HT group (p =0.1024).

Comparative analysis:

There was no significant difference between levels of vitamin D, calcium, phosphorus or parathormone when comparing the two groups. TSH concentrations were higher in patients compared to the control group as expected, with no difference in free T4. We did not find differences related to gender, alcohol consumption or cigarette smoking (Table 1).

We did not observe differences between the concentrations of interleukins (table 2).

Correlations

Hashimoto's Thyroiditis

A positive correlation was observed between vitamin D and free T4, IL-17, TNF-α and IL-5 (Table 3). There was no significant correlation between vitamin D and other immunological markers, calcium, phosphorus, PTH or TSH (data not shown).

TNF-α was positively correlated with thyroid volume. IFN-γ correlated positively with TPOAb and negatively with calcium. IL-5 and IL-17 correlated negatively with TRAb (Table 3). There was no correlation between interleukins and age, BMI, diagnostic time, parity, TSH, free T4,TPOAb, TGAb, phosphorus or PTH (data not shown).

Among these patients, 58 (66.7%) presented a non-goiter form (mean = 8.3 ml) and 29 (33.3%), the form with goiter (mean = 24.62 ml). In the subgroup with goiter there was a negative correlation of volume with IL-2 (Th1) concentrations (r = -0.47330; p = 0.0146). The volume did not correlate with other interleukins or vitamin D in both subgroups.

Control Group

In the control group, there was a negative correlation between vitamin D and age (r = -0.31374, p = 0.013) whereas there was no correlation between vitamin D and interleukins, thyroid hormone profile, calcium, phosphorus or PTH (data not shown).

Analysis of logistic regression:

In HT group, simple logistic regression analysis showed FT4 (OR = 0.063; p = 0.0066, CL95%: 0.009 - 0.464) and TNF-α (OR = 0.907, p = 0.0130, CL95%: 0.840-0.980) at lower levels as predictive factors of reduction of vitamin D levels. There were no significant values between vitamin D insufficiency and the other variables. In the multiple logistic regression analysis, lower levels of free T4 was a risk factor for vitamin D insufficiency (OR = 0.076; 95% CI: 0.008-0.764, p = 0.0286).

A receiver operator characteristic (ROC) curve was built to discriminate the best threshold for concentrations of free T4 to discriminate insufficiency and sufficiency of vitamin D. We found a threshold of 1.18 *ng/dl*, with an accuracy of 70.8%, specificity of 82.6% and sensitivity of 55.9% (figure 1).

In the control group, after simple logistic regression analysis, it was verified that, age (OR = 1.051, p = 0.0182, CL95%: 1.008 - 1.095) and IL-4 (OR = 1.013, p = 0.0415, CL95%: 1.000 – 1.026) were predictive factors of vitamin D insufficiency. There were no significant values between lower levels of vitamin D and the other variables: sex, smoking, parity, calcium, phosphorus, PTH, TSH, free T4 and other interleukins

studied. There were no risk factors in the multiple logistic regression analysis for the control group.

DISCUSSION:

The present study demonstrated that vitamin D concentrations were similar in patients HT and the control group. Similarly, D'Aurizio et al., revealed no differences in vitamin D deficiency and 25OHD levels between 100 AITDs patients (52 TH and 48 GD) and healthy controls. Goswami et al., reported no association of vitamin D deficiency and TPOAb positivity, but only a weak inverse correlation between serum 25OHD and TPOAb levels in 642 patients. Effraimids et al., developed a longitudinal study with 803 AITDs subjects who concluded that vitamin D deficiency was not associated with the early stages of thyroid autoimmunity. Yasmeh et al reported a higher prevalence of sufficient vitamin D levels in HT females relative to control, observed a significant positive correlation between vitamin D and TPOAb levels in HT males relative to control concluding that HT was not associated with vitamin D deficiency relative to the control group. Zhang et al., pointed out that Vitamin D status was not associated with positive thyroid autoantibodies in a cross-sectional study. Higher vitamin D levels were associated with lower TSH levels in males. On the other hand, Ma et al (2015) evaluated the association between vitamin D concentrations and AITDs (GD, HT and postpartum thyroiditis) in two independent case-control studies, observing a decrease in vitamin D levels in these patients when compared to controls. Meta-analysis of Wang (2015) and the Mazokopakis review (2014) also demonstrated this association. Thus, differences in the association between HT and vitamin D insufficiency remain unresolved in the literature [11,17,22-24,29-31].

We emphasize that the reduction of free T4 levels was a predictor of vitamin D insufficiency for Hashimoto's thyroiditis, especially at concentrations below 1.18 ng/dl, but not for the euthyroid individuals of the control group, suggesting that the thyroid hormone status would play a role in the maintenance of vitamin D sufficiency and its immunomodulatory role would influence the presence of thyroid autoimmune disease. Additionally, the positive correlation between free T4 and vitamin D concentrations suggests that adequate levothyroxine replacement in HT would be an

important factor in maintaining vitamin D at sufficient levels, similarly previous reports. Likewise, Bozkurt et al (2013) demonstrated a direct relationship with vitamin D in patients with recent and long-standing HT and demonstrated that the severity of vitamin D deficiency correlated positively with disease time and higher concentrations of anti-thyroid antibodies. Ma et al (2015), D'Aurizio et al (2015) and Ke et al (2017) found no association between free T4 and TSH with vitamin D insufficiency in GD and HT. Zhang et al (2014) observed in a cross-sectional study that higher concentrations of vitamin D were associated with low TSH, independent of free T4 values in euthyroid men from middle age to the elderly. Mansournia et al and Tamer at al found that vitamin D level presented decreasing trend in hypothyroidism patients who failed to administer medication. Krysiak et al (2017) finding that vitamin D administration decreased thyroid antibody titers, especially TPOAb in HT women already receiving levothyroxine treatment suggesting that vitamin D may potentiates de effect of levothyroxine on thyroid autoimmune control [12,17,23,31-35].

Additionally, lower concentrations of TNF-α was a predictor of lower levels of vitamin D, pointing to the relationship between vitamin D and the immune system. This finding not corroborates data from the literature about the direct action of TNF-α/Th-1 cytokines on the pathophysiology of HT and its presence in higher concentrations in vitamin D insufficiency but the patients in the present study had several years of established disease and were under treatment with levothyroxine, which could justify the low concentrations of TNF-α, a cytokine usually present in the disease development phase. In addition, serum cytokine levels may be different from intra thyroid levels [7,10,12,22,34,36].

There was also a positive correlation between vitamin D and TNF-α, IL-5 and IL-17. Low levels of TNF-α and IL-17 in detriment of low levels of vitamin D, unlike what is commonly described in the literature, could be justified by the control of cytotoxicity arising from the treatment of the disease since the patients of this study present in mean ten years of diagnosis and are on levothyroxine replacement therapy. Regarding IL-5, the literature has demonstrated, as in the present study, a positive association with vitamin D levels. According to Cantorna et al, vitamin D is associated with reduced production of cytokine group associated with cytotoxicity. Furthermore, a correlation was demonstrated between stimulation of autoantibodies,

as well as higher concentrations of CD8 T lymphocytes and specific cytokines in the peripheral blood of patients with intense disease activity. Marchiori et al observed reduction of pro-inflammatory cytokines in hypothyroid patients under treatment. On the other hand, Ke at al (2017) not found association with vitamin D levels and serum cytokines IL-4, IL-17 and TNF-α in patients with HT and GD [33,36,37].

In addition, we observed no association of vitamin D and thyroid volume, different from Bizzaro et al., 2015, who reported relation between low vitamin D and AITDs, anti-thyroid antibodies and higher thyroid volumes with higher levels of TSH, describing as a predisposing factor the VDR polymorphism present in cells of the immune system. Besides that, Pani et al. found polymorphisms in the vitamin D carrier protein (DBP) in GD but not in HT [38,39].

We considered as limitations of this study the small number of patients and individuals in the control group, however it was possible to obtain significant results and we reached the number recommended by the sample calculation. Blood samples were collected in the months between spring and summer, however in our region the seasons do not show significant differences for sun offered during the months.

In conclusion, our study verified that lower levels of vitamin D has not been associated with HT, however thyroxine levels were determined as a risk factor to vitamin D insufficiency. We emphasize the importance of maintaining adequate concentrations of T4 for the adequate vitamin D status in patients with HT, a fact not verified in euthyroid individuals. The association between vitamin D and TNF-α, IL-5 and IL-17 in these patients pointed to the relevance to this relationship with autoimmunity in HT.

Additional studies are warranted to clarify the precise role of vitamin D in AITD.

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None of the authors has any potential conflicts of interest associated with this research.

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Table 1: Demographic and laboratory characteristics of HT and control group [mean (min-max)*]

Characteristics	Patients HT (n =88)	Control Group (n=71)	P
Age (years)*	46.2 (20 – 66)	47.4 (19 – 77)	0.5346
Gender (F/M) (n)	82/06	61/09	0.1983
BMI (Kg/m^2)*	28.1(16.8-45.5)	26.4(17–42.6)	0.0516
Time of diagnosis (years)*	10.6 (1-47)	----------	---------
Alcohol consumption*	1 (1.2%)	0 (0%)	1.000
Smoking*	9 (11%)	12 (19%)	0.2148
FT4 (ng/dl)*	1.2(0.5-1.8)	1.2 (0.8-1.6)	0.4510
	Median: 1.2	Median: 1.2	
TSH (µUl/ml)*	6.3 (0.3-57)	2.0(0.6-5.0)	<0.0001
	Median: 3.3	Median: 1.7	
TPOAb (Ul/ml)*	571.4 (30-3000)	----------	-------
TGAb (Ul/ml)*	677.3 (100-4000)	----------	--------
TRAb (Ul/L)*	1.9 (0.2-40)	----------	--------
Calcium (mg/dl)*	9.1(7.6-10.4)	9.1(8.5-10.1)	0.9194
Phosphor (mg/dl)*	3.3(2.0-5.4)	3.4(2.5-4.5)	0.1486
PTH (pg/ml)*	41.4 (8.4-207)	38.4 (11-127.1)	0.3779
25OHVitaminD (ng/ml)*	26.4 (7.6-48.2)	28.6 (13-51.2)	0.1917
Vitamin D Insufficiency	61 (71.8%)	39 (59.1%)	0.1024

Mann-Whitney; Chi-square; Fisher exact test

Table 2: Serum concentrations of interleukins Th1 (IL-2, TNF-α, IFN-γ), Th2 (IL-4, IL-5) and Th17 (IL-17) in HT and control group [mean (min-max)]

Interleukins	**HT (n =88)**	**Control Group (n=71)**	**P**
IL-2 (pg/ml)	0.7(0.1-7.3)	0.6 (0.1-5.7)	**0.9556**
IL-4 (pg/ml)	50.4 (1-330.2)	48.0(0.9-378.3)	**0.2067**
IL-5 (pg/ml)	1.6 (0.0 -15.5)	1.6(0.0-20.8)	**0.3597**
TNF-α (pg/ml)	13.3 (0.2-35)	11.8 (0.2-35.5)	**0.0904**
IFN-γ (pg/ml)	17.0 (0.1-228)	13.4 (0.1-152)	**0.9165**
IL-17 (pg/ml)	**13.9 (0.1-176)**	**12.3(0.1-177)**	**0.6157**

Mann-Whitney test

Table 3: Correlations between interleukins, vitamin D and variables in HT. Correlation coefficients (Spearman 's rho)

		Thyroid volume	Calcium	TRAb	TPOAb	Vitamin D
IFN- γ	***r***		- 0.21906	- 0.22278	0.24208	
	p		0.0415	0.0381	0.0265	
TNF- α	***r***	0.22508				**0.37505**
	p	0.0372				**0.0004**
IL-17	***r***			- 0.26848		**0.35050**
	p			0.0119		**0.0011**
IL-5	***r***			- 0.30565		**0.26610**
	p			0.0040		**0.0144**
FT4	***r***					**0.25199**
	p					**0.0224**

***r*: correlation**

***p*: estatistical significance level**

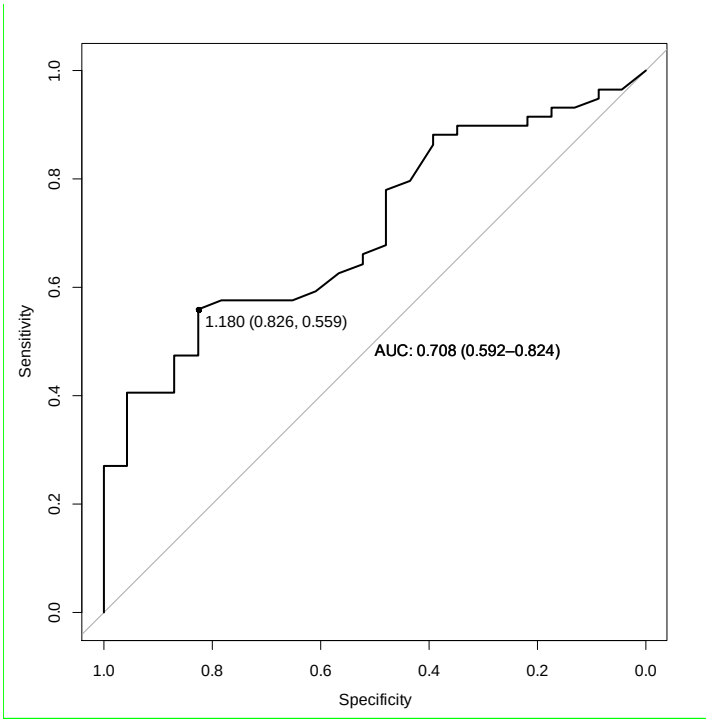

Figure 1. ROC (receiver operator characteristic) curve used to identify threshold values related to insufficiency of 25OHvitamin D (< 30 ng/ml) in patients with Hashimotos' thyroiditis. For a concentration of free thyroxine of 1.18 ng/dl, sensitivity was 55.97%, specificity was 82.6%, and accuracy was 70.8%.

DISCUSSÃO:

Evidências crescentes sobre os efeitos imunomodulatórios da vitamina D vêm surgindo nos últimos anos. Deficiência de vitamina D é um problema de saúde global. Tem-se demonstrado que até mesmo a população que vive em locais de clima tropical está sob risco de queda nos níveis de vitamina D, o que pode ser atribuído a mudanças no estilo de vida como por exemplo, menor exposição solar. A incidência de doenças autoimunes aumentou progressivamente nos últimos anos, o que motiva cada vez mais a realização de estudos sobre os efeitos da vitamina D no sistema imunológico. O presente estudo foi realizado com pacientes portadores de Tireoidite de Hashimoto (TH) e Doença de Graves (DG) nos quais estudamos interleucinas, anticorpos antitireoidianos e concentrações de vitamina D (28-30,58-61).

Avaliando 218 pacientes portadores de TH em eutireoidismo, Mazokopakis e cols (2015) demonstraram o papel da suplementação de vitamina D gerando redução nas concentrações séricas de AcTPO. Metanálise realizada por Wang e cols (2015) concluiu que a vitamina D pode desempenhar um papel no desenvolvimento das doenças tireoidianas autoimunes. Effraimidis e cols (2012) concluiu que baixas concentrações de vitamina D não se associaram a estágios iniciais das DTAs. Apesar de vários estudos envolvendo o tema, os dados da literatura permanecem conflitantes e inconclusivos. Por outro lado, D'Aurizio e cols (2015) verificaram que baixos níveis de vitamina D não se correlacionaram com TH ou DG (71,73, 89, 90).

No presente estudo os níveis de vitamina D foram significativamente menores em pacientes portadores de DG o que não aconteceu no grupo TH. Pani e cols (2002) encontraram associação entre polimorfismos no gene da proteína transportadora de vitamina D (DBP) em pacientes portadores de DG, achado este não encontrado no grupo TH. Giovinazzo e cols (2017) concluíram que baixas concentrações de vitamina D estão presentes em pacientes recém diagnosticados com TH e são inversamente proporcionais aos títulos de AcTPO, porém estudaram polimorfismos genéticos no receptor de vitamina D (VDR) e não encontraram

correlação com a manifestação da doença. Yasmeh e cols (2016) também não encontraram associação entre TH e insuficiência de vitamina D (68,70,91).

Encontramos associação direta entre vitamina D e citocinas TNF-α, IL-5 and IL-17 nos pacientes portadores de TH evidenciando a importância da correlação entre autoimunidade e vitamina D na TH. Adicionalmente, baixas concentrações de TNF-α e T4L foram fatores de risco para insuficiência de vitamina D nesses pacientes. A correlação entre vitamina D e citocinas nas doenças tireoidianas autoimunes é bem documentada na literatura como citado por Unal e cols (2014), Arslan e cols (2015), Colotta e cols (2017), entre outros. Krysiak e cols (2017) descreveram queda nos níveis de AcTPO em pacientes com TH sob tratamento com levotiroxina que receberam suplementação com vitamina D descrevendo o papel da mesma em produzir efeitos imunosupressivos nesses pacientes (21, 29, 66, 67).

A prevalência de insuficiência de vitamina D foi maior em pacientes com DG em tratamento com tionamidas comparado tanto aos pacientes sem tratamento quanto aos controles. Tal achado pode sugerir uma possível ligação entre a eficácia do tratamento e os níveis de vitamina D nesses pacientes. O bom controle da doença refletido por concentrações ideais de T4 livre pode estar associado a níveis adequados de vitamina D. No grupo DG baixas concentrações de T4 livre foi fator de risco para insuficiência de vitamina D, o que não aconteceu no grupo controle.

Yasuda e cols (2012), Ke e cols (2017), e Ma e cols (2015) e Planck e cols (2018) não encontraram associação entre perfil hormonal tireoideano e insuficiência de vitamina D na DG. Por outro lado, Ahn e cols (2017) demonstraram associação significativa entre baixos níveis de vitamina D e menor taxa de remissão da doença após suspensão do tratamento com tionamidas (33, 52, 54, 92,93).

No presente estudo IFN-γ foi preditor de insuficiência de vitamina D no grupo DG. Encontramos correlação inversa entre altas concentrações de IFN-γ e IL5 em pacientes com pouco tempo de diagnóstico, sugerindo que essa citocinas, associadas a insuficiência de vitamina D poderiam ter um papel relevante da fase inicial da doença autoimune. Adicionalmente, no grupo DG, níveis de IL-4 apresentaram correlação inversa com T4L.

O tratamento com tionamidas, realizado em parte de nossos pacientes, resultou em mediana de T4 livre normal, porém, encontramos maior frequência de

insuficiência de vitamina D. Desta forma, sugerimos haver uma possível influência das tionamidas e/ou do perfil de vitamina D nas concentrações elevadas de IL-4 em pacientes com diagnóstico inicial de DG ou com hipertireoidismo descompensado. Portanto, o tratamento da doença com drogas antitireoidianas poderia interferir na expressão de citocinas. Por outro lado existem estudos demonstrando que não há associação entre interleucinas e vitamina D em pacientes com DG (33,52, 54,90).

O desenho transversal da maioria dos estudos além de amostras pequenas, população heterogênea, variação sazonal das amostras de sangue coletadas, variabilidade analítica entres os métodos de dosagem de vitamina D, influência ou interação com fatores externos como infecção estão entre os fatos que não permitem diferenciar se a insuficiência de vitamina D resulta das alterações metabólicas da disfunção tireoidiana por si ao invés de um evento primário envolvido na patogênese da doença. Ensaios randomizados controlados multicêntricos de amostra ampla são necessários para ajudar a consolidar se existe uma associação entre vitamina D e doenças tireoidianas autoimunes e, consequentemente, fornecer melhor suporte e segurança sobre um possível efeito benéfico decorrente da suplementação de vitamina D nesses pacientes.

CONCLUSÕES:

1. Grupo Graves

- A prevalência de insuficiência de vitamina D foi maior em DG do que nos indivíduos saudáveis eutireoideos do grupo de controle.

- A redução significativa das concentrações de vitamina D especialmente nos pacientes do grupo DG em uso de tionamidas sugere que a insuficiência de vitamina D pode estar associada à fase de atividade imunológica da doença ou de descompensação da função tireoidiana ou mesmo à ação da droga.

- Menores concentrações de IFN-γ foram fator preditor de insuficiência de vitamina D demonstrando que em nossos pacientes houve relação entre esta citocina classicamente implicada no processo de autoimunidade tireoidiana e a vitamina D.

- Menores concentrações de tiroxina livre, ainda que dentro dos valores de referência, se evidenciaram como fator preditor de insuficiência de vitamina D, refletindo que, talvez, atingir níveis adequados de tiroxina facilite a manutenção de níveis recomendados de vitamina D, fato não observado nos indivíduos eutireoideos do grupo de controle.

- Menores concentrações de vitamina D associaram-se a maiores volumes tireoideanos, geralmente associados a menor chance de remissão do hipertireoidismo.

2. Grupo Hashimoto:

- A prevalência de insuficiência de vitamina D foi semelhante em TH e indivíduos saudáveis eutireoideos do grupo de controle.

- Menores níveis de tiroxina livre, ainda que dentro dos valores de referência, foram preditores de insuficiência de vitamina D nos pacientes com TH indicando a importância da manutenção do eutireoidismo no adequado *status* de vitamina D.

- Concentrações de vitamina D se correlacionaram diretamente às concentrações das interleucinas TNF-α, IL-5 e IL-17 envolvidas na fisiopatologia e no processo de autoimunidade da TH, evidenciando assim a ligação entre vitamina D e os marcadores imunológicos na Tireoidite de Hashimoto

- Apenas nos pacientes com TH e não no grupo de controle houve relação entre vitamina D e interleucinas, indicando o envolvimento entre o processo imunológico na TH e a vitamina D.

3. Contribuições do estudo:

Enfatizamos o importante papel de concentrações adequadas de tiroxina em pacientes com Doença de Graves e Tireoidite de Hashimoto para manutenção do *status* suficiente de vitamina D, fato este não verificado em indivíduos eutireoideanos do grupo controle.

Adicionalmente, podemos citar a forte evidência de correlação existente entre a fisiopatologia da Doença de Graves e a insuficiência de vitamina D.

A associação entre vitamina D e as interleucinas TNF-α, IL-5 e IL-17 nos pacientes com tireoidite de Hashimoto aponta a relevância desta correlação da vitamina D com a autoimunidade na TH.

Esperamos contribuir com o fornecimento de dados para o desenvolvimento de novos estudos sobre possíveis tratamentos futuros para estas doenças. Ainda assim, ressaltamos que estudos adicionais são necessários para elucidar o papel preciso da vitamina D em doenças tireoidianas autoimunes visto que os dados da literatura são insuficientes para estabelecer uma ligação direta entre deficiência da vitamina e o desenvolvimento de ambas as doenças.

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ANEXOS

ANEXO 1: Termo de Consentimento Livre e Esclarecido para Pesquisa em Seres Humanos

Projeto:

"VITAMINA D EM DOENÇAS TIREOIDIANAS AUTOIMUNES E RELAÇÃO COM PERFIL HORMONAL TIREOIDIANO E ATIVIDADE INFLAMATÓRIA DE CÉLULAS Th1, Th2 e Th17."

Responsáveis pela pesquisa: Profa. Dra. Denise Engelbrecht Zantut Wittmann

Dra Ilka Mara Borges Botelho

Eu,.....,HC n° ....., ...anos, portador do RG n° ....., residente à ....., n° ....., Bairro ....., cidade de ....., Estado de ....., telefone....., ou através de....., ... anos, RG nº....., residente à ..... n°....., Bairro ....., cidade de ....., Estado ....., telefone ....., grau de parentesco ....., responsável por mim, concordo em participar da realização deste protocolo, observados os itens abaixo.

OBJETIVOS E JUSTIFICATIVA: Segundo estudos recentes pacientes com baixas concentrações de vitamina D no sangue estão mais predispostos a desenvolver doença crônica da tireoide. A relação entre a deficiência de vitamina D e o desenvolvimento da doença tireoideana ainda não está muito bem definida, necessitando estudos mais aprofundados sobre o papel da vitamina D como causa ou consequência da doença em questão, bem como a relação entre reposição da mesma no controle da atividade da doença justificando a realização do presente projeto de pesquisa.

PROCEDIMENTOS A SEREM REALIZADOS: Não haverá alteração na rotina de seguimento ambulatorial nem do tratamento do paciente que participar do estudo. Além dos exames de rotina do nosso ambulatório, será colhida uma amostra de cerca de 15 ml de sangue da veia periférica para dosagem de hormônios e outras substâncias relacionadas à tireoide (T_4L, T3L, TSH, anticorpos antitireóide (AcTPO, AcTg), anticorpo antirreceptor de TSH (TRAB) e substâncias que refletem o estado de inflamação crônica que pode estar presente na doença tireoideana (IL-2, IFN-γ, TNF-α, IL-17, IL-4 e IL-5). Será realizado também um exame de Ultrassonografia da tireoide para avaliar o tamanho e textura da glândula, além de presença de nódulos na mesma. Os pacientes serão tratados, sendo mantidos o tratamento e o atendimento médico de rotina. Não haverá estoque de material biológico (do sangue colhido) para outro tipo de uso neste estudo ou em outros futuros.

ESCLARECIMENTOS : A equipe responsável pela pesquisa estará sempre pronta e preparada para esclarecer quaisquer dúvidas dos pacientes nos assuntos relacionados à pesquisa e ao tratamento, e compromete-se a proporcionar informação atualizada sobre o assunto em estudo, ainda que esta possa afetar a vontade do indivíduo em continuar participando da pesquisa.

OBS: Os dados do pesquisador são para dúvidas sobre a pesquisa, e os dados do CEP são para denúncias e/ou reclamações referentes aos aspectos éticos da Pesquisa.

O **NÃO CONSENTIMENTO** em participar do estudo ou a desistência de participar da pesquisa a qualquer tempo não irá alterar o atendimento da paciente no ambulatório. Todos os dados referentes ao indivíduo no estudo serão mantidos em sigilo e a identificação do paciente em estudo não será exposta em conclusões ou publicações posteriores.

Telefone da secretaria da Disciplina de Endocrinologia: (019) 3521-7775

Telefone da secretaria do Comitê de Ética em Pesquisa: (019) 3521-8936

Campinas, ... de ..... de .....

....

RESPONSÁVEL PELO ESTUDO **PACIENTE OU RESPONSÁVEL**

ANEXO 2: Termo de Consentimento Livre e Esclarecido para Pesquisa em Seres Humanos

GRUPO DE CONTROLE

Projeto:

"VITAMINA D EM DOENÇAS TIREOIDIANAS AUTOIMUNES E RELAÇÃO COM PERFIL HORMONAL TIREOIDIANO E ATIVIDADE INFLAMATÓRIA DE CÉLULAS Th1, Th2 e Th17."

Responsáveis pela pesquisa: Profa. Dra. Denise Engelbrecht Zantut Wittmann

Dra Ilka Mara Borges Botelho

Eu,.....,HC n° ....., ...anos, portador do RG n° ....., residente à ....., n° ....., Bairro ....., cidade de ....., Estado de ....., telefone....., ou através de....., ... anos, RG nº....., residente à ..... n°....., Bairro ....., cidade de ....., Estado ....., telefone ....., grau de parentesco ....., responsável por mim, concordo em participar da realização deste protocolo, observados os itens abaixo.

OBJETIVOS E JUSTIFICATIVA: Segundo estudos recentes pacientes com baixas concentrações de vitamina D no sangue estão mais predispostos a desenvolver doença crônica da tireoide. A relação entre a deficiência de vitamina D e o desenvolvimento da doença tireoideana ainda não está muito bem definida necessitando estudos mais aprofundados sobre o papel da vitamina D como causa ou consequência da doença em questão, bem como a relação entre reposição da mesma no controle da atividade da doença justificando a realização do presente projeto de pesquisa.

PROCEDIMENTOS A SEREM REALIZADOS: Será colhida uma amostra de cerca de 15 ml de sangue da veia periférica para dosagem de hormônios e outras substâncias relacionadas à tireoide (T_4L, T3L, TSH, anticorpos antitireóide (AcTPO, AcTg), anticorpo antirreceptor de TSH (TRAB) e substâncias que refletem o estado de inflamação crônica que pode estar presente na Tireoidite de Hashimoto (IL-2, IFN-γ, TNF-α, IL-17, IL-4 e IL-5). As dosagens servirão para formação de um grupo de controle para o estudo, composto por pessoas saudáveis. Não haverá estoque de material biológico (do sangue colhido) para outro tipo de uso neste estudo ou em outros futuros.

ESCLARECIMENTOS: A equipe responsável pela pesquisa estará sempre pronta e preparada para esclarecer quaisquer dúvidas relacionadas à pesquisa, e compromete-se a proporcionar informação atualizada sobre o assunto em estudo, ainda que esta possa afetar a vontade do indivíduo em continuar participando da pesquisa.

OBS: Os dados do pesquisador são para dúvidas sobre a pesquisa, e os dados do CEP, são para denúncias e/ou reclamações referentes aos aspectos éticos da Pesquisa.

O **NÃO CONSENTIMENTO** em participar do estudo ou a desistência de participar da pesquisa a qualquer tempo, não terá conseqüências para um eventual atendimento futuro neste serviço, nem qualquer outra implicação futura. Todos os dados referentes ao indivíduo no estudo serão mantidos em sigilo e a identificação não será exposta em conclusões ou publicações posteriores.

Telefone da secretaria da Disciplina de Endocrinologia: (019)3521-7775
Telefone da secretaria do Comitê de Ética em Pesquisa: (019)3521-8936

Campinas, ... de ..... de .....

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RESPONSÁVEL PELO ESTUDO **PACIENTE OU RESPONSÁVEL**

ANEXO 3: Parecer consubstanciado do CEP

Plataforma Brasil - Ministério da Saúde

PROJETO DE PESQUISA

Faculdade de Ciências Médicas - UNICAMP

Número do Parecer: 62370
Data da Relatoria: 10/07/2012

Versão 2
CAAE: 03330912.3.0000.5404

Área Temática:

Título: Tireoidite de Hashimoto e Deficiência de Vitamina D: estudo de prevalência e relação com autoimunidade
Pesquisador: Denise Engelbrecht Zantut Wittmann
Instituição: Faculdade de Ciências Médicas - UNICAMP

Apresentação do Projeto:

O projeto encontra-se muito bem redigido, proporcionando ao leitor uma visão clara dos objetivos propostos pela pesquisadora.

Objetivo da Pesquisa:

Este projeto tem por objetivo estudar as alterações de vitamina D em pacientes portadores de Tireoide Crônica Autoimune (Tireoide de Hashimoto) e relação com as citocinas inflamatórias envolvidas no desenvolvimento da doença.

Avaliação dos Riscos e Benefícios:

Este trabalho não possui riscos previsíveis aos pacientes, porém como a relação entre a deficiência de vitamina D e o desenvolvimento de TH ainda não está muito bem definida, este estudo poderá auxiliar a identificar a vitamina D como causa ou consequência da TH, bem como a relação entre a reposição da vitamina D no controle da doença e o papel das citocinas inflamatórias em cada condição.

Comentários e Considerações sobre a Pesquisa:

A pesquisa proposta apresenta grande relevância à comunidade científica, podendo auxiliar no tratamento de pacientes com TH.

Considerações sobre os Termos de apresentação obrigatória:

A pesquisadora apresentou todos os termos de apresentação obrigatória redigidos de acordo com as normas exigidas.

Recomendações: Sem recomendações no momemento

Conclusões ou Pendências e Lista de Inadequações: Projeto aprovado

Situação do Parecer: Aprovado

Necessita Apreciação da CONEP: Não

Conforme discussão em reunião do colegiado em 24/07/2012.

CAMPINAS, 28 de Julho de 2012

Assinado por: Carlos Eduardo Steiner

ANEXO 4: Endocrine Journal Editorial Office

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Dear Dr. Ilka Botelho

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ANEXO 5: Endocrine Journal

Endocrine Journal

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ORIGINAL

Vitamin D in Hashimoto's thyroiditis and its relationship with thyroid function and inflammatory status

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Abstract. Several studies have shown the correlation between vitamin D [25(OH)D] deficiency and thyroid autoimmunity and reducing of thyroid autoantibodies in patients with normal levels of vitamin D combining with thyroid hormone replacement. However, other authors not agree with this association. It is still unclear whether the low 25(OH)D levels are the result of HT disease or a part of its cause. We studied 88 patients with HT regarding vitamin D status and thyroid autoimmunity markers as well as the relationship with cytokines produced by Th1, Th2, and Th17 cells compared with a control group of 71 euthyroid healthy subjects. The present study demonstrated that vitamin D concentrations were similar in patients HT and the control group. The reduction of free T4 levels was a predictor of vitamin D insufficiency for Hashimoto's thyroiditis, but not for the control group. Lower concentrations of TNF-α was a predictor of lower levels of vitamin D. Differences in the association between HT and vitamin D insufficiency remain unresolved in the literature. The thyroid hormone status would play a role in the maintenance of vitamin D sufficiency, and its immunomodulatory role would influence the presence of autoimmune thyroid disease. The positive correlation between free T4 and vitamin D concentrations suggests that adequate levothyroxine replacement in HT would be an essential factor in maintaining vitamin D at sufficient levels.

Key words: Hashimoto's thyroiditis, Cytokines, Vitamin D, Autoimmunity

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HASHIMOTO'S THYROIDITIS (HT) is one of the autoimmune thyroid diseases (AITDs), also called chronic lymphocytic thyroiditis. Autoimmune attack on the thyroid plays with infiltration of the gland by T and B lymphocytes associated with thyroid peroxidase antibodies (TPO-Ab) and thyroglobulin antibodies (TG-Ab) production [1-5].

In HT occurs a genetic defect in T cell suppressor (Treg) function and CD4+ T cells are not deleted when they are free to promote activation of B-lymphocytes. Concomitantly, the Th cells produce cytokines that induce thyrocytes to express surface antigens HLA-DR making them susceptible to immune attack. There is an interaction between susceptibility genes and environmental factors associated with autoimmune dysfunction [1-3, 6].

Th1 cells secrete inflammatory cytokines such as interferon-γ (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor alpha (TNF-α), essential for the cell-mediated immune response. Th2 cells secrete the inflammatory cytokines IL-4 and IL-5, necessary for the antibody-mediated immune response. Th-17, another Th cell subtype composed of CD4+ T lymphocytes shows involvement in the pathophysiology of autoimmune diseases, especially in HT, and the production of interleukins such as IL-17 and IL-23 [2, 7-9].

HT manifests clinically in goiter or non-goiter (or atrophic) forms. The goiter form is related to the predominance of cellular immunity through activation of

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Th1 promoting apoptosis of the thyroid follicular cells leading to the dysfunction. The non-goiter form is related to the predominance of humoral immunity, *via* activation of Th2 that induces antigen-specific B lymphocytes to produce anti-TSH receptor antibodies (TRAb) stimulus blockers thus causing the disease [3, 10].

Several studies have shown the correlation between vitamin D deficiency and thyroid autoimmunity. Vitamin D3 (cholecalciferol) is produced in the skin when in the presence of ultraviolet-B radiation (UVB) and can also be obtained through supplementation and diet. There is a hydroxylation in the liver and an action of 1 alpha-hydroxylase in the kidney for product its active form calcitriol (1,25 $(OH)_2$VitD), that binds to the nuclear vitamin D receptor (VDR), which is expressed in various immune cells such as monocytes, macrophages, dendritic cells, B and T lymphocytes, promoting immunomodulatory actions. Low concentrations of vitamin D are associated with predisposition to various autoimmune diseases such as type 1 diabetes mellitus, rheumatoid arthritis, multiple sclerosis and autoimmune thyroid diseases (AITDs). There are reports on the association between low vitamin D levels and the presence of TPO-Ab as well as the association of polymorphisms in the VDR gene in patients with AITDs. Calcitriol inhibits proliferation of Th1 cells and production of cytokines as well as induces B cell apoptosis [3, 11-21].

Multiple factors influence the synthesis of vitamin D through the skin such as duration of sun exposure, latitude, season, time of day, pigmentation of the skin, use of sunscreen, behavioral habits and diet. The reference values for vitamin D concentrations in our setting were recently reviewed, being considered as vitamin D insufficiency values below 20 ng/mL [22-25].

Many studies have shown reducing of thyroid autoantibodies in patients with normal levels of vitamin D combining with thyroid hormone replacement. However, other authors not agree with this association. It is still unclear whether the low 25(OH)D levels are the result of HT disease or a part of its cause [26-28].

Considering the possible role of vitamin D in thyroid autoimmunity, we aimed to study the relationship of vitamin D with thyroid function and inflammatory *status* in patients with HT.

Methods

Study design

We studied patients with HT regarding vitamin D status and thyroid autoimmunity markers as well as the relationship with cytokines produced by Th1, Th2 and Th17 cells compared with a control group of euthyroid healthy subjects.

An interim analysis was conducted to evaluate the statistical power of correlation between thyroid hormone, vitamin D and thyroid autoimmunity markers resulting in the minimum of 88 patients. Then, we included 88 patients with HT followed in our university hospital and 71 individuals without AITD, all aged 18 to 65 years. Blood samples were collected from the two groups for measurements of serum total 25OH vitamin D, thyrotropin (TSH), free thyroxine (FT4), calcium, phosphorus, parathormone (PTH), TPOAb, TGAb, and TRAb. Cytokines produced by Th1 cells (IL-2, IFN-γ, TNF-α), Th2 (IL-4, IL-5) and Th17 (IL-17) were measured in all participants.

Ultrasound estimated thyroid volume in patients. Data on weight, height, body mass index, parity and time since direct interview collected diagnosis. Written informed consent was obtained from each patient or subject after full explanation of the purpose and nature of all procedures. The University Ethics in Research Committee approved the study.

Inclusion criteria

Only patients with the diagnosis of HT were included in the study.

The diagnosis of thyroid dysfunction was based on high concentrations of TSH and low FT4. At the time of collection, all patients with HT were on replacement therapy with levothyroxine. Only patients with high levels of antithyroid antibodies confirming the etiology of AITD were included.

All subjects in the control group were clinical and laboratory euthyroid, TPOAb, TGAb and TRAb undetectable and were selected from patient's companions or hospital staff.

Exclusion criteria

Exclusion criteria were: previous history of thyroidectomy, acutely ill patients, active malignant or inflammatory disease, use of amiodarone, steroids, calcium and/or vitamin D, use of iodinated contrast less than 3 months before the start of the study, heart failure (class III or IV NYHA), severe liver disease (reduced albumin or increased INR), advanced kidney disease (stage 4 or 5), patients under hemodialysis, seropositive for HIV or hepatitis C, and pregnant.

Laboratory evaluation of thyroid and vitamin D status

For evaluation of thyroid status, TSH was measured by electrochemiluminescence (Roche Cobas Elecsys—reference values RV 0.41 to 4.5 μUI/mL) and FT4 dosed by competitive chemiluminescence immunoassay Elecsys FT4 II (RV 0.9 and 1.8 ng/mL). The intra-assay coefficient of variation (CV) was 5%; measuring range 0.01–100 μUI/mL, analytical sensitivity 0.01 pg/mL and functional sensitivity 0.014 μUI/mL with inter-assay CV of 20%. For FT4 measurement interval was used between 0.02 to 7.76 ng/dL, intra-assay CVs of 5% analytical sensitivity 0.023 ng/mL and functional 0.39 ng/mL and inter-assay CV 20%. TPOAb and TGAb were measured by chemiluminescent immunometric assay Elecsys (RV up to 34 UI/mL and up to 115 IU/mL, respectively). For TPOAb, measurement interval was 5.0 to 600.0 IU/mL, CV 5% analytical sensitivity 5.0 IU/mL Functional 34 IU/mL. For TGAb measurement interval was between 10.0 to 4,000.0 IU/mL (5% CV); analytical sensitivity 10 IU/mL and functional sensitivity 34 IU/mL. TRAb was measured by competitive electrochemiluminescence immunoassay using Elecsys TRAb TSH receptors (RV up to 1.22 IU/L); measurement interval range from 0.3 to 40.0 IU/L, CV 5%; analytical sensitivity of 0.3 IU/L and functional sensitivity of 0.9 IU/L. Total vitamin D (25OHVitD) was measured by the test LIAISON® 25 OH Vitamin D TOTAL using chemiluminescent immunoassay technology (CLIA) for the quantitative determination of 25-hydroxyvitamin D and other hydroxylated metabolites of vitamin D in human serum, plasma or EDTA plasma with lithium heparin using the evaluation of the amount of vitamin D using the family LIAISON® analyzers.

We adopted the following recommendations for TSH levels: patients <60 years old 1.0–2.5 mU/L; between 60–70 years 3–4 mU/L and >70 years 4–6 mU/L [29].

Vitamin D levels between 30 and 60 ng/mL are recommended for at-risk groups as patients with autoimmune diseases and were adopted as adequate to the study population [22].

Cytokines evaluation

Cytokines were measured by the immunoassay technique of Milliplex Map based on the Luminex Xmap technology. Luminex uses proprietary techniques to internally color-code microspheres with two fluorescent dyes. Through precise concentrations of these dyes, distinctly colored bead sets polystyrene microspheres or magnetic microspheres can be created, each of which is coated with a specific capture antibody. The reference values of cytokines are described below: IL-2 RV 1.0–1.6 pg/mL; INF-γ RV 0.8–1.1 pg/mL; TNF-α RV 0.7–1.1 pg/mL; IL-4 RV 4.5–7.1 pg/mL; IL-5 RV 0.5–0.7 pg/mL; IL-17 RV 0.7–1.2 pg/mL.

Thyroid ultrasound evaluation

The total thyroid volume was determined in milliliters (mL) by the product of the longitudinal, transverse and anteroposterior measurements multiplied by the constant 0.52, adding up the volumes of the right and left lobes and isthmus. Values between 6 and 15 mL (10–11 ± 3–4 mL) were considered normal for adults [30].

Statistical methods

Exploratory data analysis was performed through summary measures (frequency, percentage, mean, standard deviation, minimum, median and maximum). The groups were compared using Kruskal-Wallis, Chi-Square or Fisher's exact test. The correlation of Vitamin D and Interleukins with the other variables was evaluated using the Spearman coefficient or the Mann-Whitney test. Factors associated with vitamin D deficiency were assessed through logistic regression using the *stepwise* selection criteria. The significance level for statistical analysis was 5% [31-33].

Results

Descriptive analysis

The study included 159 participants, 88 patients with HT, of which 82 were female (93%). In the control group, there were 71 subjects, 61 female (85.9%). Mean-time of diagnosis of HT was ten years (range 1–47 years). Clinical and laboratory characteristics of patients and control groups are described in Table 1.

Vitamin D levels below 30 ng/dL were found in 59.1% (n = 39) of the control group and in 71.8% (n = 61) of HT group (p = 0.1024).

Comparative analysis

There was no significant difference between levels of vitamin D, calcium, phosphorus or parathormone when comparing the two groups. TSH concentrations were higher in patients compared to the control group as expected, with no difference in free T4. We did not find differences related to gender, alcohol consumption or cigarette smoking (Table 1).

Table 1 Demographic and laboratory characteristics of HT and control group [mean (min–max)*]

Characteristics	Patients HT (n = 88)	Control Group (n = 71)	p
Age (years)*	46.2 (20–66)	47.4 (19–77)	0.5346
Gender (F/M) (n)	82/06	61/09	0.1983
BMI (Kg/m^2)*	28.1 (16.8–45.5)	26.4 (17–42.6)	0.0516
Time of diagnosis (years)*	10.6 (1–47)	—	—
Alcohol consumption*	1 (1.2%)	0 (0%)	1.000
Smoking*	9 (11%)	12 (19%)	0.2148
FT4 (ng/dL)*	1.2 (0.5–1.8) Median: 1.2	1.2 (0.8–1.6) Median: 1.2	0.4510
TSH (μUI/mL)*	6.3 (0.3–57) Median: 3.3	2.0 (0.6–5.0) Median: 1.7	<0.0001
TPOAb (UI/mL)*	571.4 (30–3,000)	—	—
TGAb (UI/mL)*	677.3 (100–4,000)	—	—
TRAb (UI/L)*	1.9 (0.2–40)	—	—
Calcium (mg/dL)*	9.1 (7.6–10.4)	9.1 (8.5–10.1)	0.9194
Phosphor (mg/dL)*	3.3 (2.0–5.4)	3.4 (2.5–4.5)	0.1486
PTH (pg/mL)*	41.4 (8.4–207)	38.4 (11–127.1)	0.3779
25OHVitaminD (ng/mL)*	26.4 (7.6–48.2)	28.6 (13–51.2)	0.1917
Vitamin D Insufficiency	61 (71.8%)	39 (59.1%)	0.1024

Mann-Whitney; Chi-square; Fisher exact test

Table 2 Serum concentrations of interleukins Th1 (IL-2, TNF-α, IFN-γ), Th2 (IL-4, IL-5) and Th17 (IL-17) in HT and control group [mean (min–max)]

Interleukins	HT (n = 88)	Control Group (n = 71)	p
IL-2 (pg/mL)	0.7 (0.1–7.3)	0.6 (0.1–5.7)	0.9556
IL-4 (pg/mL)	50.4 (1–330.2)	48.0 (0.9–378.3)	0.2067
IL-5 (pg/mL)	1.6 (0.0–15.5)	1.6 (0.0–20.8)	0.3597
TNF-α (pg/mL)	13.3 (0.2–35)	11.8 (0.2–35.5)	0.0904
IFN-γ (pg/mL)	17.0 (0.1–228)	13.4 (0.1–152)	0.9165
IL-17 (pg/mL)	13.9 (0.1–176)	12.3 (0.1–177)	0.6157

Mann-Whitney test

We did not observe differences between the concentrations of interleukins (Table 2).

Correlations

Hashimoto's Thyroiditis

A positive correlation was observed between vitamin D and free T4, IL-17, TNF-α and IL-5 (Table 3). There was no significant correlation between vitamin D and other immunological markers, phosphorus or TSH (data not shown). We also found no correlation between vitamin D and PTH (r = –0.07044; p = 0.5373).

TNF-α was positively correlated with thyroid volume. IFN-γ correlated positively with TPOAb and negatively with calcium. IL-5 and IL-17 correlated negatively with TRAb (Table 3). There was no correlation between interleukins and age, BMI, diagnostic time, parity, TSH, free T4, TPOAb, TGAb, phosphorus or PTH (data not shown).

Among these patients, 58 (66.7%) presented a non-goiter form (*mean* = 8.3 mL) and 29 (33.3%), the form with goiter (*mean* = 24.62 mL). In the subgroup with goiter there was a negative correlation of volume with IL-2 (Th1) concentrations (r = –0.47330; p = 0.0146). The volume did not correlate with other interleukins or vitamin D in both subgroups.

Control Group

In the control group, there was a negative correlation between vitamin D and age (r = –0.31374, p = 0.013) whereas there was no correlation between vitamin D and interleukins, thyroid hormone profile or phosphorus

Table 3 Correlations between interleukins, vitamin D and variables in HT. Correlation coefficients (Spearman's rho)

		Thyroid volume	Calcium	TRAb	TPOAb	Vitamin D
IFN-γ	*r*		–0.21906	–0.22278	0.24208	
	p		0.0415	0.0381	0.0265	
TNF-α	*r*	0.22508				0.37505
	p	0.0372				0.0004
IL-17	*r*			–0.26848		0.3505
	p			0.0119		0.0011
IL-5	*r*			–0.30565		0.2661
	p			0.004		0.0144
FT4	*r*					0.25199
	p					0.0224

r: correlation *p*: estatistical significance level

(data not shown). Still, we did not find correlation between vitamin D and PTH in the controls (r = –0.16112; p = 0.1998).

Analysis of logistic regression

In HT group, simple logistic regression analysis showed FT4 (OR = 0.063; p = 0.0066, CL 95%: 0.009–0.464) and TNF-α (OR = 0.907, p = 0.0130, CL 95%: 0.840–0.980) at lower levels as predictive factors of reduction of vitamin D levels. There were no significant values between vitamin D insufficiency and the other variables. In the multiple logistic regression analysis, lower levels of free T4 was a risk factor for vitamin D insufficiency (OR = 0.076; 95% CI: 0.008–0.764, p = 0.0286).

A receiver operator characteristic (ROC) curve was built to discriminate the best threshold for concentrations of free T4 to discriminate insufficiency and sufficiency of vitamin D. We found a threshold of 1.18 ng/dL, with an accuracy of 70.8%, specificity of 82.6% and sensitivity of 55.9% (Fig. 1).

In the control group, after simple logistic regression analysis, it was verified that, age (OR = 1.051, p = 0.0182, CL 95%: 1.008–1.095) and IL-4 (OR = 1.013, p = 0.0415, CL 95%: 1.000–1.026) were predictive factors of vitamin D insufficiency. There were no significant values between lower levels of vitamin D and the other variables: sex, smoking, parity, calcium, phosphorus, PTH, TSH, free T4 and other interleukins studied. There were no risk factors in the multiple logistic regression analysis for the control group.

Discussion

The present study demonstrated that vitamin D concentrations were similar in patients HT and the control group. Similarly, D'Aurizio *et al.* revealed no differences in vitamin D deficiency and 25OHD levels between 100 AITDs patients (52 TH and 48 GD) and healthy controls. Goswami *et al.* reported no association of vitamin D deficiency and TPOAb positivity, but only a weak inverse correlation between serum 25OHD and TPOAb levels in 642 patients. Effraimids *et al.* developed a longitudinal study of 803 AITDs subjects who concluded that vitamin D deficiency was not associated with the early stages of thyroid autoimmunity. Yasmeh *et al.* reported a higher prevalence of sufficient vitamin D levels in HT females relative to control, observed a significant positive correlation between vitamin D and TPOAb levels in HT males relative to control concluding that HT was not associated with vitamin D deficiency relative to the control group. Zhang *et al.* pointed out that Vitamin D status was not associated with positive thyroid autoantibodies in a cross-sectional study. Higher vitamin D levels were associated with lower TSH levels in males. On the other hand, Ma *et al.* (2015) evaluated the association between vitamin D concentrations and AITDs (GD, HT, and postpartum thyroiditis) in two independent case-control studies, observing a decrease in vitamin D levels in these patients when compared to controls. Meta-analysis of Wang (2015) and the Mazokopakis review (2014) also demonstrated this association. Thus, differences in the association between HT and vitamin D

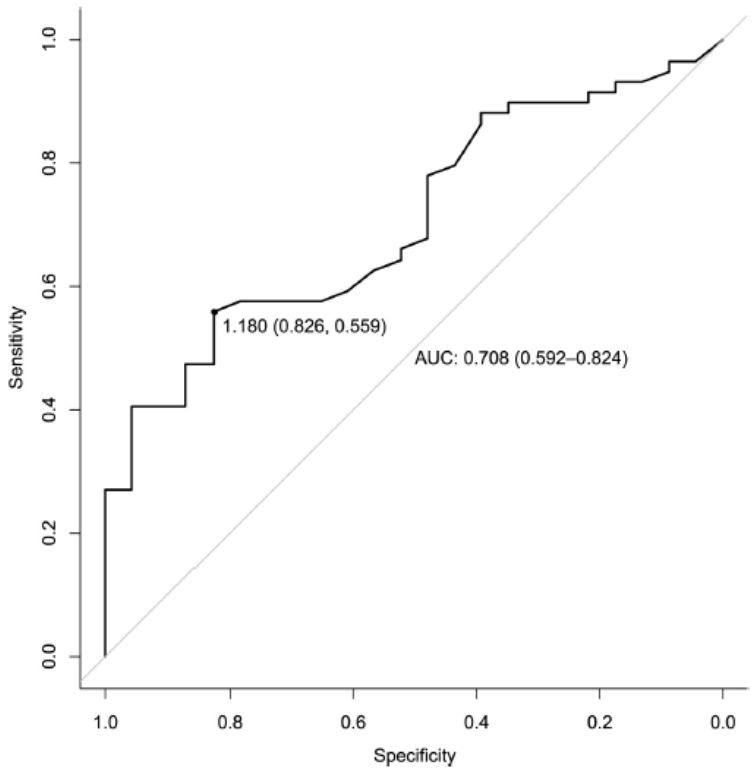

Fig. 1 ROC (receiver operator characteristic) curve used to identify threshold values related to insufficiency of 25OHvitamin D (<30 ng/mL) in patients with Hashimotos' thyroiditis. For a concentration of free thyroxine of 1.18 ng/dL, sensitivity was 55.97%, specificity was 82.6%, and accuracy was 70.8%.

insufficiency remain unresolved in the literature [11, 17, 26-28, 34-36].

We emphasize that the reduction of free T4 levels was a predictor of vitamin D insufficiency for Hashimoto's thyroiditis, especially at concentrations below 1.18 ng/dL, but not for the euthyroid individuals of the control group, suggesting that the thyroid hormone status would play a role in the maintenance of vitamin D sufficiency, and its immunomodulatory role would influence the presence of autoimmune thyroid disease. Additionally, the positive correlation between free T4 and vitamin D concentrations suggests that adequate levothyroxine replacement in HT would be an essential factor in maintaining vitamin D at sufficient levels, similarly previous reports. Likewise, Bozkurt *et al.* (2013) demonstrated a direct relationship with vitamin D in patients with recent and long-standing HT and demonstrated that the severity of vitamin D deficiency correlated positively with disease time and higher concentrations of anti-thyroid antibodies. Ma *et al.* (2015), D'Aurizio *et al.* (2015) and Ke *et al.* (2017) found no association between free T4 and TSH with vitamin D insufficiency in GD and HT. Zhang *et al.* (2014) observed in a cross-sectional study that higher concentrations of vitamin D were associated with low TSH, independent of free T4 values in euthyroid men from middle age to the elderly. Mansournia *et al.* and Tamer *et al.* found that vitamin D level presented decreasing trend in hypothyroidism patients who failed to administer medication. Krysiak *et al.* (2017) finding that vitamin D administration decreased thyroid antibody titers, especially TPOAb in HT women already receiving levothyroxine treatment suggesting that vitamin D may potentiate the effect of levothyroxine on autoimmune thyroid control [12, 17, 27, 36-40].

Additionally, lower concentrations of TNF-α was a predictor of lower levels of vitamin D, pointing to the

relationship between vitamin D and the immune system. This finding not corroborates data from the literature about the direct action of TNF-α/Th-1 cytokines on the pathophysiology of HT and its presence in higher concentrations in vitamin D insufficiency. However, the patients in the present study had several years of established disease and were under treatment with levothyroxine, which could justify the low concentrations of TNF-α, a cytokine usually present in the disease development phase. Also, serum cytokine levels may be different from the levels within the thyroid cells [7, 10, 12, 26, 39, 41].

There was also a positive correlation between vitamin D and TNF-α, IL-5, and IL-17. Low levels of TNF-α and IL-17 in detriment of low levels of vitamin D, different from the literature description, could be justified by the control of cytotoxicity arising from the treatment of the disease since the patients of this study present in mean ten years of diagnosis and are on levothyroxine replacement therapy. Regarding IL-5, the literature has demonstrated, as in the present study, a positive association with vitamin D levels. According to Cantorna *et al.*, vitamin D is associated with reduced production of cytokine group associated with cytotoxicity. Furthermore, a correlation was demonstrated between stimulation of autoantibodies, as well as higher concentrations of CD8 T lymphocytes and specific cytokines in the peripheral blood of patients with intense disease activity. Marchiori *et al.* observed reduction of pro-inflammatory cytokines in hypothyroid patients under treatment. On the other hand, Ke *et al.* (2017) not found an association with vitamin D levels and serum cytokines IL-4, IL-17 and TNF-α in patients with HT and GD [38, 41, 42].

In addition, we observed no association of vitamin D and thyroid volume, different from Bizzaro *et al.*, 2015, who reported relation between low vitamin D and AITDs, anti-thyroid antibodies and higher thyroid volumes with higher levels of TSH, describing as a predisposing factor the VDR polymorphism present in cells of the immune system. Besides that, Pani *et al.* found polymorphisms in the vitamin D carrier protein (DBP) in GD but not in HT [43, 44].

Many researchers have been studied the single nucleotide polymorphisms (SNPs) in vitamin D receptor (VDR) to identify genes involved in the pathogenesis of autoimmune thyroid diseases (AITDs). Among the known polymorphisms related to the VDR locus are FokI, BsmI, ApaI, and TaqI. Ban *et al.* described the presence of VDR polymorphisms in both HT and GD besides the association between VDR-FokI polymorphism and risk of osteoporosis in GD. On the other hand, Giovinazzo *et al.* found no relationship between VDR polymorphisms and the presence of HT unlike Lin W *et al.* who described a correlation between VDR-FokI polymorphism and the risk of developing TH [20, 45-48].

Interestingly, the correlation between vitamin D and PTH did not reach significance in both patients and controls. This finding may be justified due to the mean values of vitamin D in both groups were above 25 ng/mL, not affecting calcium levels in general. Also, there was no correlation with vitamin D in both the patients' group and the control group. Although studies have shown that vitamin D levels below 30 ng/mL could lead to an increase in PTH, this did not happen in the present study [19, 49, 50].

We considered as limitations of this study the small number of patients and individuals in the control group. However, it was possible to obtain significant results and we reached the number recommended by the sample calculation. Blood samples were collected in the months between spring and summer, however, in our region, the seasons do not show significant differences for sun offered during the months.

In conclusion, our study verified that lower levels of vitamin D have not been associated with HT, however thyroxine levels were determined as a risk factor for vitamin D insufficiency. We emphasize the importance of maintaining adequate concentrations of free T4 for the adequate vitamin D status in patients with HT, a fact not verified in euthyroid individuals. The association between vitamin D and TNF-α, IL-5 and IL-17 in these patients pointed to the relevance to this relationship with autoimmunity in HT.

Additional studies are warranted to clarify the precise role of vitamin D in AITD.

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Disclosure

None of the authors has any potential conflicts of interest associated with this research.

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